Summer 1998

Characterization of the Biological Functions of Human Recombinant Zona Pellucida Protein 3

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CHARACTERIZATION OF THE BIOLOGICAL FUNCTIONS OF HUMAN RECOMBINANT ZONA PELLUCIDA PROTEIN 3

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A Dissertation submitted to the Faculty of
Old Dominion University and
Eastern Virginia Medical School in Partial Fulfillment of the
Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCES

OLD DOMINION UNIVERSITY and
EASTERN VIRGINIA MEDICAL SCHOOL
August 1998

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ABSTRACT

CHARACTERIZATION OF THE BIOLOGICAL FUNCTIONS OF HUMAN RECOMBINANT ZONA PELLUCIDA PROTEIN 3

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Recombinant human zona pellucida protein 3 (rhZP3), expressed, isolated and purified from PA-1 cells, is characterized for its biological activity and the role in the signal transduction pathway. Characterization of the biological activity of rhZP3 was detected by hemizona assay and immunofluorescence staining of acrosome reaction. The results indicated that rhZP3 exhibited an inhibition in the binding assay (HZI 43.6 ± 3.3; n=9; 30 ng/mL rhZP3) and induction of acrosome reaction (198.6% ± 77.2% increase from baseline; n=29; 30 ng/mL rhZP3). It was further confirmed by the transmission electron microscopy that there was no difference in morphology of rhZP3-induced, acrosome-reacted spermatozoa and the calcium ionophore A23187-induced, acrosome-reacted spermatozoa. Recombinant human zona pellucida protein 3 also exhibited a dose-dependency in both inhibition of the binding assay and immunofluorescence staining for acrosome reaction. Furthermore, the antagonistic action of pertussis toxin on the G_i-protein resulted in a decrease in the stimulation of acrosome reaction by the rhZP3. Also, there were no detectable changes in $[Ca^{2+}]_i$ in the rhZP3-treated spermatozoa as well as in the potentiation study of progesterone and rhZP3 by FURA-2 spectrofluorometry.

Hence the present study concludes that rhZP3 is both a binding ligand for the sperm-zona pellucida interaction and acrosome reaction inducer. It is deduced that the optimal experimental conditions of the rhZP3 in the immunofluorescence staining of acrosome reaction are a concentration of rhZP3 protein of at least 30 ng/mL, capacitation time of 4 hours, and 0.5-4 million spermatozoa per mL. In addition, the acrosome reaction induced by the rhZP3 utilizes G_i-protein dependent pathway. Finally, there are no changes in $[Ca^{2+}]_i$ detected in populations of sperm with use of FURA-2 spectrofluorometry.

I wish to dedicate this dissertation to my parents

Chung Keng Juan and Rue Yu Hu

Who are always in a constant support of my education.

ACKNOWLEDGMENTS

I wish to thank my advisors, Drs. Sergio Oehninger and Ke-Wen Dong, who guided me throughout the project. I also want to thank Dr. Mary Mahony for her support and help as well as the committee members, Drs. Frank Castora, Mark Elliott, and Michael Solhaug for their efforts in advising in this project. Finally, I want to include a special thanks to Dr. Peter Blackmore who contributed his time and knowledge in seeking the role of rhZP3 in the signal transduction pathway of acrosome reaction.

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INTRODUCTION

A. Fertilization

Fertilization, the process whereby individual gametes from the female and male unite to create a zygote whose genetic makeup is different from both parents, occurs in the ampullary region of the fallopian tube or oviduct in the human. While spermatozoa can stay alive in the female reproductive tract for about 24 hours, the oocyte is thought to degenerate 12 to 24 hours after ovulation, if not fertilized (Shettles, 1970).

Fertilization proceeds as a well-choreographed cascade of cell-to-cell interactions involving cell-to-cell adhesion, fusion, and signaling events between gametes of opposite mating types (Yanagimachi, 1994; Wassarman, 1995).

The initial interaction of mammalian spermatozoa and oocytes goes through steps of adhesion and recognition, primary binding, as well as secondary binding. Adhesion of sperm head to the oocyte is a loose, non-specific association of the plasma membrane with the zona pellucida followed by a species-specific recognition. Primary binding is a relatively strong interaction between capacitated acrosome-intact spermatozoa and the zona pellucida mediated by ZP3 and its complementary sperm receptor leading to tight binding. The secondary binding is mediated by ZP2 and proacrosin / acrosin located on the inner acrosomal membrane of acrosome-reacted spermatozoa (Yanagimachi, 1981).

The Journal Model used for this dissertation was The Molecular Human Reproduction

Fertilization involves three phases for the union of sperm and oocyte. Phase 1 is the penetration of the corona radiata by the spermatozoa (Shettles. 1970). Phase 2 is the penetration of zona pellucida. The penetrability of the zona pellucida changes when the head of the spermatozoon comes in contact with the oocyte surface. This results in the release of substances that cause an alteration in the properties of the zona pellucida (the zona reaction) and inactivate species-specific receptor sites of spermatozoa (Baranska et al., 1975). Finally, phase 3 is the fusion of oocyte-sperm membranes. Since the plasma membrane covering the acrosomal head cap has disappeared during the acrosome reaction, the actual fusion is accomplished between the oocyte membrane and the membrane that covers the posterior region of the sperm head. In the human both the head and the tail of the spermatozoon enter the cytoplasm of the oocyte of the oocyte, but the plasma membrane is left behind on the oocyte surface (Yanagimachi, 1977).

The fate of the spermatocyte following oocyte penetration is as follows. First, the oocyte undergoes cortical and zona reactions to release cortical oocyte granules. Hence the oocyte membrane becomes impenetrable to other spermatozoa, and the zona pellucida alters its structure and composition through removal of zona pellucida protein receptor sites for spermatozoa. Second, the oocyte finishes its second meiotic division immediately after entry of the spermatozoon. One of the daughter cells, known as the second polar body, receives hardly any cytoplasm; the other daughter cell is the definitive oocyte. Its chromosomes (22 + X) become arranged in a vesicular nucleus known as the female pronucleus. Third, the postfusion activation may be

considered to encompass the initial cellular and molecular events associated with early embryogenesis (Yanagimachi, 1977).

From the standpoint of their genome, oocytes and spermatozoa are equal, but their life history and behavior before and during fertilization are quite different. Since both oocyte and spermatocyte are involved in the process of fertilization, their formation, physiological structures. and biochemical importance are discussed.

B. Oocytes

1. oogenesis

The mature female germ cells are derived from the primordial germ cells in the third week of embryo development (Witschj, 1948). Once the primordial germ cells have arrived in the gonad of a genetic female they differentiate into oogonia. The majority of the oogonia continue to divide by mitosis but some of them differentiate into primary oocytes. Immediately after their formation. they replicate their DNA and enter the prophase of the first meiotic division (Manotaya and Potter, 1963). Approximately at the time of birth. all primary oocytes have finished the prophase of the first meiotic division and their number is estimated to vary from 700,000 to 2,000,000 (Weakley. 1966). But instead of proceeding into metaphase, they enter the dictyotene stage, a resting stage during prophase in response to the oocyte maturation-inhibiting factor secreted by the granulosa cells, a single layer of cells that surrounds the oocyte, until the onset of puberty during which a number of primordial follicles begin to mature with each ovarian cycle.

The female hormone system has three hierarchies of hormones: a hypothalamic releasing hormone, gonadotropin-releasing hormone (GnRH), the anterior pituitary hormones, follicle-stimulating hormone (FSH) and leuteinizing hormone (LH), as well as the ovarian hormones, estrogens and progesterone. These hormones are not secreted in constant amount throughout the female monthly sexual cycle in humans, but instead are secreted at differing rates during different phases of the cycle (Beyer and Feder, 1987).

In the beginning of the puberty, in respond to the increasing secretion of FSH, largely, and also LH, the primary oocyte (still in the dictyotene stage) begins to increase in size, while the surrounding epithelial cells, the follicular cells, change from flat to cuboidal, to form the primary follicle. The single-layer granulosa cells also proliferate giving rise to more layers. Also, many spindle cells in the ovarian interstitum accumulate outside the granulosa layers to form two sublayers, the theca externa, the highly vascular connective tissue layer, and the theca interna, the epitheloid layer. Soon a layer of acellular material consisting of mucopolysaccharides is deposited on the surface of the oocyte. This material, which is produced by the follicular cells as well as the oocyte, gradually increases in thickness, thus forming the zona pellucida (Zamboni, 1974). Subsequently, fluid-filled spaces coalesce to form the follicular antrum. As development continues the follicular cells surrounding the oocyte remain intact and form the cumulus oophorus. At maturity, the follicle is known as the tertiary or vesicular follicle. The follicle then has a diameter varying from 6 to 12 mm. The theca interna is considered a main source of estrogen which is secreted into the follicle causing the granuolosa cells to form more FSH

receptors; this causes a positive feedback as the vesicular follicles are more sensitive to the FSH than ever. The FSH from anterior pituitary and the estrogens from theca interna combined stimulate the production of LH receptor on the granulosa cells resulting in a very rapid increase in follicular proliferation and secretion (Karsch, 1987; Gruhn and Kazer, 1989).

About a week or more of growth, but before the ovulation, all except one follicles, the selected follicle, will undergo atresia. The surviving follicle, secretes more estrogen than others, is more advanced in developing stage. Hence, it will be allowed to develop into the mature follicle. Hence this causes the FSH to enhance the proliferation of granulosa and thecal cells as well as the co-stimulation of FSH and estrogen to promote the increasing numbers of both FSH and LH receptors on the granulosa cells and to a lesser extent on the thecal cells (Karsch, 1987). As soon as the follicle is mature, the primary oocyte resumes its first meiotic division, leading to formation of two daughter cells of unequal size, but each with 2n DNA. One cell, the secondary oocyte, receives all of the cytoplasm; the other, the first polar body, receives practically none. Then the secondary oocyte enters the second meiotic division giving 1n DNA. The moment the secondary oocyte shows the spindle formation, ovulation occurs and the oocyte is shed from the ovary (Gruhn and Kazer, 1989).

Ovulation in the human takes place 14 days after the onset of menstruation in a 28-day cycle (Leung and Armstrong, 1980). There is a ovulatory surge of LH shortly before the ovulation. The LH surge causes follicle swelling and finally leads to expulsion of the ovum from the follicle. The remaining granulosa cells and thecal cells become the corpus luteum which

secretes estrogen and progesterone. In the unpregnant woman, both estrogen and progesterone have strong negative feedback on the anterior pituitary gland of decreasing the secretion of both LH and FSH. As a result, the corpus luteum begins to degenerate on approximately the 26th day of the normal cycle. This in turn removes the negative feedback inhibition of estrogen and progesterone resetting the secretion of FSH and LH in the cycle. The FSH and LH initiate growth of new follicles to start a new ovarian cycle (Mahesh, 1985).

2. Structure and biochemistry of an oocyte

At ovulation, the oocyte is surrounded by the cumulus oophorus, which consists of cumulus cells and the matrix (Yanagimachi, 1988). It is reported that only capacitated spermatozoa with intact acrosomes are able to enter the cumulus (White et al., 1990). Hence, the cumulus oophorus may serve as a barrier for uncapacitated spermatozoa and it was reported that some factors in the cumulus may be responsible to induce the acrosome reaction and stimulate sperm motility (Siiteri, et al., 1988; Tesarik, 1989).

The mammalian oocyte is also surrounded by a thick glycoprotein coat of zona pellucida under the cumulus oophorus. The entry of spermatozoa into the zona pellucida of an oocyte is assisted by the combination of mechanical action of hyperactivation and the enzymatic action of enzymes released from the acrosome-reacted sperm (Yanagimachi, 1988).

Immediately underneath the zona pellucida is perivitelline space followed by the plasma membrane or oolemma of oocyte. Cortical granules, manufactured by the Golgi apparatus during oogenesis and meiotic maturation, in mammalian

oocyte were mostly present in the coarse granular cytoplasm near the periphery (Cherr et al., 1988). In mammals, cortical granule exocytosis or cortical reaction begins in a wavelike propagation along the periphery of the oocyte. The negatively charged oolemma is also depolarized preventing additional sperm fusion until cortical gruanule exocytosis is completed. This is the primary block to polyspermy. Part of the cortical granule protein remains on the oolemma to form a hyaline layer, but other cortical granule components contribute to the elevation of the vitelline coat, and to removal of sperm-binding receptors from the vitelline envelope. Cortical granule peroxidase hardens the elevated vitelline envelope by cross-linking tyrosine residue of the vitelline coat protein giving a secondary block to polyspermy (Austin, 1956, Austin and Braden, 1956).

C. Zona Pellucida Proteins

1. Function

The zona pellucida is a highly glycosylated extracellular matrix that plays four important roles during development: (a) to regulate endocrine traffic during folliculogenesis; (b) to form a barrier to heterospecific fertilization; (c) to form a block to polyspermic fertilization; and (d) to protect the embryo during the preimplantation stage. To the sperm, the zona represents the last physical barrier prior to reaching the oocyte. (Bronson and McLaren, 1970; Keenan et al., 1991; Yamagimachi, 1994; Liu et al., 1996).

2. Structure and biochemistry

In the mouse oocyte, three glycoproteins compose the zona pellucida are

well characterized. They are zona pellucida protein 1 (ZP1), pellucida protein 2 (ZP2), and pellucida protein 3 (ZP3). SDS-PAGE revealed the heterogeneity of these glycoproteins indicating post-translational modification or glycosylation of the polypeptide backbone (Bleil and Wassarman, 1980; Wassarman, 1990a, 1990b). The molecular weights and pI's of each zona pellucida proteins in human are ZP1 (MW=80-92 KDa, pI=4.9-5.9), ZP2 (MW=58-66 KDa, pI=5.0-6.0), $ZP3_L$ (MW=54-62 KDa, pI=3.5-5.1), and $ZP3_H$ (MW=58-72 KDa, pI=3.5-5.1) (Bercegeay et al., 1995). In the zona moiety, the dimerization of ZP2 and ZP3 with cross-linkage with ZP1 forms a net-like intertwining filamental structure that act as a ligand responsible for induction of acrosome reaction (Wassarman, 1988). Each ZP2/ZP3 heteropolymer filament has a signal peptide that directs the ZP secretion. Also each ZP2 and ZP3 polypeptide possesses a very little α-helical structure and a highly hydrophobic region at its carboxyl terminus which contributes to the recognizable structural repeat (Liang et al., 1990). As a result of the three-dimensional structure and characteristic, we can ascribe barrier and recognition functions to the carbohydrates of the zona pellucida (Liang and Dean, 1993). It must be noted that the common biological property of the zona proteins detected is in soluble form. It is also reported that zona pellucida protein synthesis is below detectable in the ovulated oocytes (Liang et al., 1990; Liang and Dean, 1993).

Among the three zona pellucida proteins, ZP3 is the key role in specific primary sperm binding to the zona pellucida of ovum. Furthermore, ZP3 also induces the acrosome reaction of the sperm (Wassarman, 1990b). The genetic and molecular information of the zona pellucida proteins have been documented

including the isolation and characterization of cDNA clone of human ZP3 from the loci of the human genome (Chamberline and Dean, 1990).

Dean also (1992) reported the human ZP3 has a polypeptide backbone of 424 amino acids with *N*-linked and *O*-linked oligosaccharide side chains. *N*- and *O*- linked oligosacharides are glycans that are linked to zona proteins through the nitrogen ('*N*-linked') of asparagine and through the b-hydroxyl groups ('*O*-linked') of serine and threonine. *N*-linked and *O*-linked oligosaccharides contribute to the heterogeneity of zona glycoproteins (Wassarman. 1991). The sialic acid on glycoproteins may contribute to the relatively low isoelectric point of the zona glycoproteins. The molecular weight of human ZP3 core-protein is 47,032 Da which is composed of 8% basic amino derivatives, 12% acidic amino derivatives, 7% aromatic amino derivatives, and 32% hydrophobic residues (Chamberlin and Dean, 1990). A hydrophobic region of 26 amino acid located near the C-terminal of the ZP3 core-protein may play a role in the intracellular trafficking and the extracellular matrix interaction (Dean. 1992).

N-linked glycans share a common biosynthetic pathway of glycosylation. A presynthesized oligosaccharide template $Glc_3Man_9GlcNAc_2$ is co-translationally transferred from dolichyldiphosphate to the nascent polypetide chain of the zona protein in the endoplasmic reticulum. The protein, after being transported to the Golgi apparatus, undergoes a process of trimming and addition to give a high mannose oligosaccharide complex. The resulting *N*-linked glycans can be identified from the consensus sequence Asn-X-Ser/Thr (Struck and Lennarz, 1980). The role of *N*-linked glycans is in the regulation of protein conformation through the folding of nascent polypeptide chains and

stabilization of mature glycoproteins.

O-linked oligosaccharides, on the other hand, occurs only in the Golgi apparatus. There is no lipid-coupled oligosaccharide precursor but instead the covalent attachment of *N*-acetylgalactosamine to an acceptor amino acid is through an a1 linkage in the *O*-linked glycosylation pathway (Wilson, 1991). In mouse and possibly other mammals, *O*-linked glycans alone account for the zona's sperm receptor activity (Florman and Wassarman, 1985). Oehninger and his colleagues (1990 and 1991a) have shown that various complex moieties and lectins effectively block tight binding of human spermatozoa to homologous oocytes *in vitro* indicating the carbohydrate chains regulate sperm binding. Further studies using recombinant ZP3 (rZP3) concluded (a) human rZP3 must be appropriately glycosylated to be biologically active (Barratt et al., 1994); (b) *O*-linked glycosylation is essential in the processing of human rZP3 (van Duin et al., 1994); (c) *O*-linked oligosaccharide ligands direct pig sperm binding to ZP3 (Dostalova et al., 1995); and (d) deglycosylated pig ZP3 is unable to stimulate the production of antibodies that block sperm-zona binding *in vitro* (Berger et al., 1989).

The primary structure of ZP3 across different mammalian species exhibits a high degree of homology. These conserved regions of the primary structure of ZP3 may be attributed to part of the tertiary structure of ZP3. The variable regions in the primary structure of ZP3 may mediate the species-specific spermatozoa-oocyte interaction. C- and N-terminal domains of the ZP3 may be for the sperm-oocyte binding site and the zona pellucida filament assembly respectively. A hinge region, with two proline-rich regions (a.a. 219-230 and a.a.

255-260), connecting C- and N-terminal expand from a.a. 219 to a.a. 260 (Wassarman et al., 1995).

Spermatozoa, particularly those of man, do not readily adhere to the oocytes of foreign species. The functions of human zona pellucida proteins are to modulate the species-specific fertilization (Oehninger et al., 1993).

D. Spermatozoa

1. Spermatogenesis

Differentiation of the primordial germ cells in the female begins in the third month of development, but it begins in the male at puberty. At the time of birth, germ cells can be recognized in the sex cords of the testis as large, pale cells, surrounded by the supporting cells. The latter probably derived from the surface epithelium of the gland in the same manner as the follicular cells, become the sustentacular or Sertoli cells (Bedford et al., 1973).

The primordial germ cells give rise to the spermatogonia which in turn differentiate into the primary spermatocytes (2n). After replicating their DNA (4n) these cells then start with the prophase of their first meiotic division. Upon completion of the prophase, which lasts about 16 days, the cell gives rise to two secondary spermatocytes (2n). These cells begin immediately with their second meiotic division which results in the production of four spermatids (1n). Hence, the spermatid contains 22+X or 22+Y chromosomes. The spermatids undergo a series of changes, during a process called spermiogenesis, resulting in the production of the spermatozoa. These changes include (a) formation of the acrosome, which extends over half the nuclear surface; (b) condensation of the

nucleus; (c) formation of neck, mid-piece, and tail; and (d) shedding of majority of the cytoplasm. In human, the time required for a spermatogonium to develop into a mature spermatozoon is 61 days (Bedford et al., 1973). When fully formed, the spermatozoa enter the lumen of the seminiferous tubules. From there, they are pushed toward the epididymis by the contractile elements in the wall of the seminiferous tubules. Although initially only slightly motile, the spermatozoa obtain full motility in the epididymis (Bedford et al., 1973).

2. Structure and Biochemistry of spermatozoa

Normal human spermatozoa have three main parts head, neck, and tail. The sperm head is flattened, pyriform shape that measures 4.5 mm in length, 3 mm in width, and 1.5 mm in thickness (Kruger et al., 1996), approximately half the size of a red blood cell. The outermost layer of sperm head is the plasma membrane. Immediately follow the plasma membrane is the outer acrosomal membrane. The inner acrosomal membrane is situated below the acrosome. Within the sperm head, there are three parts namely nucleus, acrosome, and nuclear envelope. Mature spermatozoa do not have much cytoplasm as it has been shedded during the spermiogenesis. The nucleus, with condensed chromatin and nuclear vacuoles, occupies the entire head. The anterior two-third of the sperm head is covered by a cap-like acrosome and the posterior acrosomeless one-third, known as the postacrosomal region, is separated from the anterior two-third by a furrow called the equatorial region (Bedford et al., 1973; Pedersen and Fawcett, 1976).

The acrosome is a membrane-bound, cap-like structure covering the anterior portion of the sperm nucleus. The acrosome contains hydrolyzing enzymes which function to assist sperm passage through the associated cells and membrane of oocyte. The acrosome contains a number of different hydrolyzing enzymes including hyaluronidase, Neuraminidase, acrosin, acid phosphatase, etc (Zaneveld and De Jonge, 1991).

Nuclear membrane has three morphologically distinct portions namely anterior, redundant, and implantation portion. The anterior portion covers the nucleus and is restricted posteriorly by the posterior ring. The plasma membrane and the outer and inner nuclear membrane will fuse in the circumferential furrow. The redundant portion is a short fold formed by the nuclear membrane after chromatin condensation. The implantation portion is formed of the recurrent limbs of the fold which fuse at the base of the nucleus (Pedersen and Fawcett, 1976).

The sperm neck which connects sperm head and tail is a complex of cross-striated columns that terminate anteriorly with the basal plate. The sperm tail leads from the neck has three regions: the midpiece, the principle piece and the terminal piece. Along the entire length of the sperm tail, is a contractile unit called axoneme which has a 9+2 pattern of microtubules. The sperm tail is packed with mitochondria in the mid-piece, which supply ATP-derived energy for motility of the sperm (Bedford et al., 1973; Pedersen and Fawcett, 1976).

3. Several Sperm Proteins are candidates ZP Adhesion/Signaling Molecules

Having been identified as a key adhesion molecule on mouse eggs, ZP3

has become the focus through which investigators have sought to identify sperm surface proteins responsible for zona pellucida adhesion. Several sperm proteins are candidates of zona pellucida adhesion and signaling molecules. Many features of ZP3 have helped to define the criteria that these sperm proteins must possess: the zona pellucida adhesion must be on the surface of live acrosome-intact sperm and they must bind to ZP3. Zona pellucida adhesion molecules should display species specificity in binding, and antibodies or ZP3 itself should interfere with adhesion. These criteria do not suggest the additional possibility that proteins involved in binding to the zona pellucida may also be involved in triggering the acrosome reaction (Hynes, 1992; Clark and Brugge, 1995).

To date three proteins have emerged as candidate ZP3 adhesion molecules on acrosome-intact mouse sperm: a b-1,4-galactosyl transferase (Miller et al., 1992; Gong et al., 1995); a 56 KD ZP3-binding protein (Bleil and Wasssarman, 1990); and a tyrosine-phosphorylated protein, p95 (Leyton and Saling, 1989). The spermatozoa pass rapidly from the vagina into the uterus and subsequently into the uterine tubes. This ascent is probably caused by contractions of the musculature of the uterus and the fallopian tube as well as the beating ciliary movement in the lumen of the fallopian tube. Spermatozoa, upon arrival in the female genital tract, are not capable of fertilizing the oocyte. They must undergo capacitation and the acrosome reaction (Baranska et al., 1975; Yanagimachi, 1977).

E. Capacitation

1. Role of capacitation

Although the spermatozoa are "mature" when they leave the epididymis, nevertheless, their activity is still held in check by multiple inhibitory factors secreted by the genital duct epithelia. Therefore, when they are first expelled in the semen, they are still unable to perform their duties in fertilizing the ovum (Yanagimachi, 1994; Wassarman, 1995). However, on coming in contact with the fluids of the female genital tract, multiple changes occur that activate the sperm for the final process of fertilization. These collective changes are called capacitation of the spermatozoa. This process will then enable the binding of sperm to zona pellucida of the ovum more feasible which subsequently leads to fertilization (Saling and Bedford, 1981; Rao et al, 1984).

When sperm are deposited in the vagina, capacitation begins as the sperm swim through the cervical mucus which may involve in the removal of sperm-surface materials such as seminal plasma proteins (Gould, et al, 1985; and Katz et al, 1986). In mammals, capacitation takes place within the lower segment of the isthmus where the sperm is stored. While the successful capacitation of sperm occurs most efficiently after its sequential exposure to the uterus and oviduct, studies in many species including human indicate that capacitation is not organ- or species-specific (DeMayo et al, 1980; Funaki et al, 1983; and Verkuyl, 1988).

2. Events that occurs during capacitation

There is a membrane change in spermatozooa during the capacitation.

The followings are an excerpt of the events.

a. Changes in intracellular ions: It is still unclear if changes occur in the intracellular concentrations of K^+, Na^+, or Ca^{2+} during capacitation(Babcock. 1983). It is well established that a massive influx of Ca^{2+} takes place during the acrosome reaction (Yanagimachi. 1988), but little is known about the kinetics of intracellular Ca^{2+} during capacitation.

b. Changes in metabolism: Sperm may exhibit increased metabolism (e.g. glycolytic activity and oxygen consumption) during capacitation (Fraser and Ahuja,1988; Fraser and Lane,1987).

c. Changes in adenylate cyclase-cAMP systems: An increase in sperm adenylate cyclase activity during capacitation has been reported which may result in increased cAMP and turnover rate, stimulating cAMP dependent protein kinase (Stein and Fraser, 1984). Stimulated protein kinase may then alter the structure of sperm proteins through phosphorylation (Hyne, Edwards. and Smith, 1985).

d. Changes in the acrosome: Acrosome enzymes may be converted to active forms during capacitation (Goodpasture. Reddy, and Zaneveld, 1981).

e. Changes in the plasma membrane: The sperm plasma membrane is a lipid bilayer that is composed of high concentrations of sterols. including cholesterol, and phospholipids. Dispersed throughout are transmembrane and peripheral proteins and oligosaccharide chains, associated with lipids or proteins. During capacitation, membrane cholesterol decreases altering the cholesterol/phospholipid ratio. Hence, it increases membrane fluidity (Parkes and Ehrenwald, 1990).

The sperm plasma membrane undergoes a variety of changes in preparation for fertilization. The sperm plasma membrane is "biologically frozen" when spermatozoa leave the male's body and its capacitation represents "defrosting".

Following penetration of the mass of cumulus cells surrounding the oocyte (Lin et al, 1994) and gaining access to the surface of oocyte, the remaining steps of fertilization can be completed. In mammals, spermatozoon-oocyte interaction after capacitation can be subdivided into five steps: 1) acrosome-intact spermatozoa bind to the zona pellucida of oocyte; 2) interaction with the zona pellucida signals via zona pellucida proteins to undergo the acrosome reaction, a regulated exocytic event that is essential for subsequent steps; 3) sperm penetration of zona pellucida; 4) binding and fusion of plasma membranes of sperm and oocyte; and finally 5) previous events "activate" the egg to initiate zygotic development and to become refractory to the overtures of additional sperm.

The acrosome reaction occurs in the immediate vicinity of the oocyte under influence of substances emanating from the corona radiata cells and the oocyte.

F. Acrosome Reaction

Acrosome reaction refers to a phenomenon when plasma membrane of the acrosome of a living spermatozoa forms multiple fusions with the outer acrosome membrane giving fenestrated membrane fragments where the content of the acrosome is released from the site of fusion--an action called exocytosis.

Fusions usually are found in the border between the cap and the equatorial segment. It is important to note that capacitation must occur before acrosome reaction. Acrosome reaction is not reversible as compared to capacitation which is reversible. A true acrosome reaction must be occur when spermatozoa are still living and motile (Yanagimachi, 1994).

The acrosome of the mammalian sperm undergoes exocytosis shortly before fertilization. This event is required for sperm penetration through the egg's zona pellucida, and for fusion with the egg plasma membrane. A true acrosome reaction in the spermatozoa utilizes a cascade of signal transduction activities that trigger multi-fusions between the outer acrosomal membrane and overlying plasma membrane, which enables the content of the acrosome to escape through the fenestrated membranes.

Several signal transductions and second messenger systems have been proposed as candidates for ZP3-mediated actions in mouse sperm cells. These include the G_i protein, Ca^{2+}-calmodulin, adenylyl cyclase-cAMP, guanylyl cyclase-cGMP, tyrosine kinase and phospholipase-diacylglycerol-inositol triphosphate (Leyton and Saling, 1989; Kopf, 1990; Jones 1990; Lee et al., 1992). These systems may induce protein phosphorylation leading to acrosome reaction through the activation of the protein kinase A, G, and C (Kopf, 1990).

Therefore, an inability to complete the acrosome reaction may be a result of the inability to progress through the signal transduction pathway. Immature spermatozoa obtained from the proximal regions of the epididymis shows both decreased adenylyl cyclase activity (White and Aitken,1989) and decreased cAMP-dependent protein kinase activity (Hoskins et al., 1974). When the

spermatozoa exposed to either progesterone or zona proteins that leads to acrosome reaction exhibits an auto-phosphorylation on tyrosine residues of a 94 KDa membrane protein (Tesarik et al., 1993; and Naz et al., 1991).

Saling and her colleagues (1979) and Bleil and Wassarman (1983) reported that mouse zona pellucida induces the acrosome reaction of capacitated mouse spermatozoa. Many investigations were thereafter conducted in rat (Shalgi et al., 1989), hamster (Cherr et al, 1986), rabbit (O'Rand and Fisher, 1987), cattle (Florman and First, 1988), pig (Berger et al, 1989), sheep(Crozet and Dumont, 1984), monkey(Vandevoort et al., 1992), and human(Cross et al, 1988; Coddington et al., 1990).

The matrix of the zona pellucida of the oocyte in the mouse consists of zona glycoproteins. One of them is ZP3 which acts as a ligand to bind the ZP3 receptor on the plasma membrane of spermatozoa that in turn triggers a cascade of reactions leading to the acrosome reaction (Bleil and Wassarman, 1983). Before capacitation, the membrane of sperm is non-fluid and non-reactive due to its high concentration of cholesterol content in the membrane and decapacitation factor(s) attach to the zona receptor. The later causes the demobilization and confinement of the zona receptors in the plasma membrane. As capacitation occurs in the spermatozoa, the plasma membrane becomes more fluid by cholesterol and decapacitation factor(s) (DF) extrusion. Hence the zona receptors are free to move around within the membrane (Saling, 1989). Kopf and Gerton (1991) proposed that some of these zona receptors have tyrosine kinase activity. As the ZP3 ligand binds to its zona receptor, the ZP3-ZP3 receptor complex is activated that in turn stimulates a G-protein of class

G_i1 and G_i2 in the plasma membrane. The activated G_i protein then activates the phospholipase C (PLC) which then causes the cleavage of phosphatidylinositol diphosphate (PIP_2) into inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 will cause an increase intracellular Ca^{2+} from the release of Ca^{2+} storage in ER. In addition, DAG will activate Ca^{2+}-dependent protein kinase C (PKC) which will phosphrylate IP_3 to form IP_4 which regulates the voltage-gated calcium channels allowing influx of extracellular Ca^{2+}. The Ca^{2+} can act on membrane phospholipids to directly facilitate the membrane fusion by neutralizing the negative charge of membrane (Leyton and Saling, 1989; Fraser, 1994; Ward et al, 1994). The Ca^{2+} is also fusogenic by neutralizing a negative charge of membranes causing phase transition and phase separation of membrane phospholipids (Yanagimachi, 1981).

The activated G protein also stimulates phospholipases A_2 (PLA) and phospholipase D (PLD). The former cleaves phosphatidyl choline (PC) into lysophosphatidyl choline (LC) plus arachidonic acid (AA) which are fusogenic whereas the latter cleaves PC to choline and phosphatidic acid (PA) (Flemming and Yanagimachi, 1981).

Yanagimachi (1981) also suggested that the activated G protein can activate adenyl cyclase which in turn stimulates cAMP production. Then cAMP will activate cAMP-dependent protein kinase A (PKA). Also cAMP will bind and cause the Na^+-gated channel to allow the influx of Na^+ in exchange of efflux of H^+. Hence the pH of intracellular will increase.

The above mentioned processes, that is the production of fusogenic factors, an increase in intracellular Ca^{2+}, and an increase in the intracellular pH.

will trigger the acrosome reaction of a spermatozoa.

G. Hyperactivation

Hyperactivation was first observed in the sperm of the golden hamster (Yanagimachi, 1970). Hyperactivation occurs before spermundergo the acrosome reaction. It is the activity of sperm having nonprogressive (dancing) movement of the sperm head tracing an erratic figure-eight pattern with brief episodes of linear (dashing) movements. It is important to note that hyperactivation is the increased thrust produced by the sperm flagellum not a particular pattern of movement. The pattern of hyperactivity varies between species and is affected by the surrounding microenvironment and viscosity of the medium (Storey and Kopf, 1991). Hyperactivation begins sometime before or after the sperm leave the isthmus in rabbit. Hyperactivated motility may assist the spermatozoa in breaking free from the isthmus reservoir (Overstret and Cooper, 1980).

It has been demonstrated that extracellular calcium is required for hyperactivation in the hamster. Sperm exposed to calcium-free medium do not undergo capacitation and sperm removed from calcium-rich and placed in calcium-free medium lose their hyperactivation (Yanagimachi, 1982). Intracellular Ca^{2+} concentration was found to be higher in hyperactivated than in nonhyperactivated spermatozoa (Surarz et al., 1993).

CHAPTER II

STATEMENT OF PURPOSE

A. Significance of the Proposed Research

About 30-40% of infertility cases can be attributed to male reproductive dysfunction. In male patients with infertility of unknown etiology. an abnormal sperm-zona pellucida interaction is not uncommon and is correlated with the reduced sperm fertility capacity (Oehninger et. al.. 1991b.1997). Thorough understanding of human sperm-oocyte interaction will offer improved and physio-pathologically directed therapy to these patients. Therefore. there is a fundamental need to gain a deeper understanding of human sperm-oocyte interaction at its molecular and cellular level.

Given the role of mouse ZP3 in mediating fertilization in the mouse. there is considerable interest in determining its function in the human species. The physiology of human sperm-zona pellucida interaction and the pathophysiology of clinical disorders affecting human fertilization could be significantly advanced if studies could be carried out using a rhZP3. So far. these studies have been hindered by the difficulty in obtaining sufficient amounts of ZP3 from native sources. Several studies in human using solubilized human zona pellucida to study the acrosome reaction and the binding capacity of sperm-oocyte interaction have been reported (Shabanowitz and O'Rand. 1989; Cross, et. al.. 1988; Franken et al., 1996). The data could not be conclusively identify whether human ZP3 or other factors are involved in the binding capacity and induction of acrosome reaction. Production of purified. glycosylated ZP3 in a biologically

active form is fraught with technical difficulties (van Duin et al., 1994, Barratt and Hornby, 1995). Reliable and repeatable systems need to be established so that these proteins can be made available, in large quantities, for research and clinical medicine. The glycosylated rhZP3 protein produced in our laboratory was expressed in the human ovarian teratocarcinoma cell line, PA-1. Purification of rhZP3 was accomplished by a nickel-chelating affinity chromatography using a polyhistidine tail-tag at the C-terminal of the rhZP3. This expression and purification system allows the *in vitro* characterization of the biological functions of rhZP3 possible in the present study. With the advancement in expression and purification of the rhZP3 and thorough understanding of its biological functions, it can be used as a diagnostic tool to identify the male-factor infertile patients due to the unknown etiologies. It is the hope of the present study that rhZP3 can act as a key to unlock the mystery in the field of the reproductive research leading to a full understanding of its mechanism in the cellular and molecular levels.

B. Rationale of the Proposed Research

The zona pellucida protein 3 plays an important role in the primary binding and in the induction of the acrosome reaction. The binding of sperm to the zona pellucida is an essential step that leads to fertilization (Wassarman, 1988; Wassarman et al., 1995). There is limited direct evidence to support the investigation of the effect of rhZP3 on the primary binding and the acrosome reaction due to the difficulty in obtaining sufficient amount of recombinant human zona pellucida proteins. Reports on the investigation of the primary binding and

acrosome reaction are not conclusive (Shabanowitz and O'Rand, 1989; Cross. et. al., 1988; Franken et al., 1996) since they could not isolate human ZP3 as a single isolated factor in the testing.

In the mouse, it has been documented that the sperm-binding and zona-induced acrosome reaction are two independent processes. It has been speculated that there are differences in the concentration dependence of ZP3 to express sperm-binding and zona-induced acrosome reaction activity (Kopf. 1990). The concentration response curve for ZP3 zona-induced acrosome reactivity is shifted to the right of the concentration response curve for ZP3 sperm-binding activity. Hence, a proposed hypothesis (Kopf, 1990) states that successful sperm binding to ZP3 requires the interaction with one single ligand of the ZP3 molecule and that multiple ligand complex of the ZP3 molecule are required to induce a complete acrosome reaction (Wassarman, 1990; Saling, 1989; and Saling, 1991).

In order to identify and evaluate the biological activity of rhZP3 expressed and purified, two experimental procedures are carried out here namely the hemizona assay and immunofluoresence staining of acrosome reaction. The characterization of rhZP3 will also act as an auxiliary test for the biological functions of rhZP3 in relation to the choice of experimental conditions in the expression and purification system used in our laboratory employed.

Burkman *et al* (1988) reported a bioassay, the hemizona assay (HZA), that utilizes bisected, nonviable human oocytes to investigate the ability of sperm to bind to the zona pellucida in comparing the function of a known, fertile sperm sample (control) with an experimental sample (test). The specificity of the

interaction between human spermatozoa and the human zona pellucida in the HZA are being documented by the fact that the sperm tightly bound are acrosome reacted (Coddington et al.. 1990). These results provide a strong support of using HZA as a test of choice for human gamete interaction of both sperm-zona binding and zona-induced acrosome reaction (Oehninger et al.. 1993). The hemizona assay gives a distinctive testing for the sperm-binding activity of rhZP3. It is a homologous, internally-controlled assay where competition studies between the natural zona pellucida and the recombinant zona pellucida protein can be performed to achieve the statistical significance. There are three advantages of HZA: 1) the two halves or hemizonae are functionally equal surfaces that allow a controlled comparison of binding and reproducible measurements from a single oocyte; 2) the limited number of available oocytes is amplified because an internally controlled test can be performed on a single egg; and, 3) because the oocyte is split microsurgically. even fresh oocytes cannot lead to in advertent fertilization and pre-embryo formation. Dose-response and time-course studies will be conducted. The treatment of rhZP3 can also be tested for whether or not it is a true acrosome reaction inducer because of its significance in correlation to the primary sperm-binding that eventually lead to fertilization *in vivo* (Franken et al., 1991). More than 80% of sperm bound to the zona under hemizona assay conditions are acrosome reacted (as confirmed by electromicroscopy) and less than 10% of non-bound sperm or sperm in the negative control droplets are acrosome-reacted (Franken, 1991a). Therefore, it is justified to use acrosome reaction as the index for primary sperm binding. For the hemizona assay, it is reported that

the sperm-binding capability of oocytes previously inseminated at the time of in vitro fertilization but not fertilized (or inseminated-unfertilized) is similar to that of non-inseminated oocyte. Nevertheless, inseminated-fertilized but non-cleaved oocyte have almost negligible binding (Franken et al., 1991).

The exposure of rhZP3 to spermatozoa for the induction of acrosome reaction can also be detected by immunofluoresence staining of acrosome status of the sperm. The present study will evaluate rhZP3 for with various testing conditions such as dose concentration, time course, sperm concentration and capacitation period.

The present study was intended to investigate the role of rhZP3 in the induction of a physiologic acrosome reaction. We can also use protein ligands to bind zona receptors to induce acrosome reaction. This is because the zona receptors are activated and aggregated by these protein ligands which will undergo the reaction cascade as described in the previous section of acrosome reaction mechanism (Coddington et al., 1990). Some physiologic complex such as progesterone and zona proteins can also induce the acrosome reaction by binding and aggregating steroid receptors in the plasma membrane of the spermatozoa. The receptors will induce a reaction cascade causing a Ca^{2+} influx and released from the storage as well as Cl^- influx (Blackmore et al., 1991). The mechanism of the effect of the calcium ionophore A23187 as a pharmacologic inducer on the plasma membrane is to form a channel on the plasma membrane for the passage of Ca^{2+} into the intracellular space of acrosome by diffusion. The Ca^{2+} can act on membrane phospholipids (Yanagimachi, 1994).

To study the role of the rhZP3 in the signal transduction of the acrosome reaction of sperm comparing control and rhZP3-treated samples can be done. The use of pertussis toxin, an antagonist of G-protein, can detect whether the acrosome reaction will be inhibited due to the incompleteness of signal transduction pathway in the spermatozoa.

C. Specific Aims

The hypothesis of this study is that sperm-oocyte interaction requires zona pellucida protein 3 (ZP3) both as sperm-oocyte binding ligand and acrosome reaction inducer. The latter will lead to a signal transduction resulting in an acrosome reaction. Hence, the overall objective of this study is to characterize the biological activity of a recombinant human zona pellucida protein 3 (rhZP3) and to examine the signal transduction pathway of the acrosome reaction.

This project also acts as the continuation of rhZP3 research in the emphasis on the biological functions. Therefore the following specific aims are proposed:

Specific Aim 1: To characterize the biological activity of the rhZP3 as sperm-binding ligand and acrosome reaction inducer.

1a: To evaluate rhZP3 both as sperm-zona binding ligand and acrosome reaction inducer.

1b: To characterize the biological activity in terms of its dose-dependency, capacitation-dependency and effect of sperm

concentration.

1c: To study the role of rhZP3 in the induction of a "physiologic" acrosome reaction both in basal conditions and in combination with other physiologic (solubilized zona pellucida) and pharmacologic (calcium ionophore A23187) inducers. Then to evaluate whether a true acrosome reaction occurs in response to the rhZP3 treatment as evaluated by transmission electromicroscopy.

Specific Aim 2: To study the role of the rhZP3 in the signal transduction of the acrosome reaction of sperm.

2a: To evaluate the effect of rhZP3 on G-proteins by using a G-protein antagonist. pertussis toxin.

2b: To evaluate the intracellular Ca^{2+} concentration of sperm in response to rhZP3.

CHAPTER III

EXPERIMENTAL TECHNIQUES

A. Experimental Design

1. *In vitro* expression, isolation, and purification of recombinanth human Zona Pellucida Protein 3

In order to better understand the recombinant human ZP3 used in the present study, an excerpt of its isolation, expression and purification based on Chi's work (1998) was included. An mRNA extract was isolated from human ovarian tissue. RT-PCR (Reverse Transcriptase - Polymerase Chain Reaction) was performed to reverse-transcribe the hZP3 mRNA into hZP3 cDNA which was subcloned into the pBluescript II SK(+) plasmid (PSK) successfully in our laboratory (Chen, 1995). Then generate a 1.3 kbp fragment containing the full length of hZP3-cDNA with a six histidine tail and a XhoI restriction site from the hZP3 PSK.

The pcDNA3.1(+) plasmid (Invitrogen, Carlsbad, CA), contains human cytomegalovirus (CMV) immediate-early promoter/enhancer for high-level expression in a variety of mammalian cell lines, was used as a vector to generate transfected PA-1 cell line. The ligation-products were transformed into E. coli cells and were selected on ampicilline LB agar (Sigma, St. Loius, MO) at 37°C. The purified DNA was examined by both restriction endonuclease digestion and polymerase chain reaction to confirm the insertion of the hZP3-sixHis DNA into the pcDNA3.1(+) vector. Also, the Sequenase Version 2.0 DNA Sequencing Kit (Amersham, Piscataway, NJ) was used to perform DNA

sequencing.

Stable Transfection of PA1 cells with hZP3-sixHis-pcDNA3.1(+) DNA was performed by the calcium phosphate-mediated transfection method (Sambrook et al., 1989). After culturing and collecting the cells, they were lysed and the cell lysate was collected from the culture plates and was subjected to the extraction of DNA. To confirm the expression of hZP3-sixHis cDNA in stable-transfected PA-1 cell, use RT-PCR and gel electrophoresis.

The transfected PA-1 cells were cultured and expressed. The rhZP3 was secreted into the media which was collected for purification. Purification of rhZP3 started with Agarose-wheat germ agglutinin (WGA) (Vector Laboratories. Inc., Burlingame, CA) in a 4°C cold room. Glycoproteins binding to the WGA resin were separated into two elution peaks with elution buffer A (10 mM PBS. pH 7.4, 0.15 M NaCl, 20 mM N-acetyl-D-glucosamine (Sigma) and elution buffer B (10 mM PBS, pH 7.4, 0.15 M NaCl, 500 mM N-acetyl-D-glucosamine).

At this point, the glycoprotein eluants were subjected to two different purification strategies namely: purification of hZP3 by DEAE - cellulose anion exchanger and non-denaturing purification of His-tagged rhZP3.

The condition for the DEAE-cellulose anion exchange purification is described as follows. Ten grams of DEAE - Cellulose resin (Sigma) was suspended in 200 mL of 0.1N NaOH for 10 minutes at room temperature. The resin was then filter on a glass funnel and washed with at lease 20 volumes of dioionized water. The resin was then suspend in 200 mL of 0.1N H_3PO_4 (Fisher Scientific, Hampton, NH) for 10 minutes at room temperature. The resins were then washed with D.W. until the filtrate reached neutral pH. The DEAE -

Cellulose was then suspended in 200 ~ 300 mL of ion exchange binding buffer (5 mM Tris-Phosphate buffer, pH 8.5) and stored at 4° C with daily changes until pH was equilibrated. The treated DEAE - Cellulose resin was suspended in ion exchanger binding buffer and let the gel settle by gravity. The column was equilibrated in the binding buffer by washing with 10 column volume of binding buffer. 30 mL of glycoproteins fraction, isolated from the cultured cell media with the WGA affinity chromatography, was applied to DEAE - Cellulose column (1 x 5 cm) while collecting flow through in sterile tube and then apply flow through into the column again. The column was then washed with binding buffer until the A_{280} was less than 0.01. The elution was accomplished by using binding buffer containing different concentration of NaCl. The elute which stayed within the peak of 280 nm absorbance was collected. Glycerol (Fisher) was added into the purified protein sample to a 50% final concentration and then frozen with liquid nitrogen (-196°C) and then stored at -80°C for acrosome reaction and hemizona assay to test the biological activity of purified rhZP3.

The conditions for the non-denaturing purification of His-tagged glycoprotein are illustrated as follows. The histidine tagged glycoprotein (rhZP3) was purified from the glycoprotein fraction, isolated from the cultured cell media with WGA affinity chromatography, with Ni-NTA (nitrilo-tri-acetic acid) resin (Qiagen). Proteins containing one or more 6xHis affinity tags, located at either the amino or carboxyl terminus of the protein, bind to the Ni-NTA resin with an affinity ($Kd=10^{-13}$ at pH 8.0) far greater than the affinity between most antibodies and antigens, or enzymes and substrates (Hoffmann and Roeder, 1991; Janknecht et al., 1991). A 1 mL of completely resuspended 50% slurry was

transferred into column and then washed with 5 resin-volume of H_2O. The resin was equilibrated with 10 resin-volume of Ni-NTA binding buffer. The WGA isolated glycoprotein samples dialyzed against Ni-NTA binding buffer were passed through an equilibrated Ni-NTA column containing 0.5 mL resin. The flow rate was adjusted at 3 ~ 4 resin volume per hour. After passing all the glycoprotein sample through the Ni-NTA column, the resin was washed with 10 resin volume of Ni-NTA binding buffer containing 0.4% Tween 20 (Fisher) and then Ni-NTA washing buffer (50 mM PBS, pH 6.6, 300 mM NaCl) until the flow-through A_{280} was less than 0.01. His-tagged glycoproteins were eluted from the resin with Ni-NTA washing buffer containing different concentration of imidazole (Sigma). (Due to the pending patent, only partial conditions are revealed). Glycerol (Fisher) was added into the purified protein sample to a 50% final concentration and then frozen with liquid nitrogen (-196°C) and then store at -80°C for acrosome reaction and hemizona assay to test the biological activity of purified rhZP3. The BCA protein assay (Pierce, Rockford, IL) was used to measure the protein concentration of the purified protein samples. In addition, SDS-PAGE electrophoresis and Western blotting using a rabbit polyclonal antibody generated against a synthetic decapeptide (D-V-T-V-G-P-L-I-F-L) of hZP3 were performed to detect and confirm the presence of the rhZP3.

2. Hemizona assay (HZA): Sperm-Zona Binding Test

Narishige micromanipulators (Tokyo, Japan) mounted on a phase-contrast inverted microscope (Nikon Diaphot, Garden City, NY) were utilized to cut the oocyte into two matching halves or hemizonae. One hemizona was

transferred to the control droplet whereas its corresponding matching hemizona was transferred to the pre-treatment droplet of rhZP3 (Chi. 1998). Gametes were incubated for 3 hours at 5% CO_2, 37°C after 1 hour swim-up and 30 minute rhZP3 treatment post-swimup. After 3-hour incubation period, the hemizonae were rinsed in culture medium by means of a finely drawn glass pipette to dislodge loosely attached sperm. The number of spermatozoa tightly bound to the convex surface of each hemizona was counted by phase-contrast microscope at 600x magnification. For each assay, the HZA results were expressed as hemizona index or HZI that was calculated as the ratio of number of sperm bound for treatment and number of sperm bound for control multiply by 100.

In addition, experiments were also tested with control sperm in Ham's F-10 / 0.5% Human Serum Albumin (Irvine Scientific, Santa Ana, CA) versus test sperm exposed to the different cell transfection and protein separation conditions mentioned earlier. Each experiment was done with 3 matching hemizonae and the HZI of each was averaged to get the final score of HZI.

3. Hemizona assay (HZA): Dose-dependency studies

The conditions used for the dose-response studies were exactly like the section of "Hemizona assay (HZA): Sperm-Zona Binding Test." The tested hemizonae were exposed to various concentrations of rhZP3 (10, 30, 100, 250, 500, and 2000 ng/mL). Three matching pairs of hemizonae were scored for Hemizona Indices that were averaged and reported.

4. Immunofluorescence techniques for the detection of acrosome reaction

A probe of fluorescein isothiocyanate (FITC)-conjugated *Pisum satvum* agglutinin (PSA) (Vector Lab, Burlingame, CA) was used to evaluate the acrosomal status of spermatozoa in spot slides. The same slides were counterstained with Hoechst 33258 stain, a DNA-specific stain, which enters the nuclear membrane of dead spermatozoa to give a fluorescent counterstain. The epiflurorescent microscope was used to evaluate the spot slides at a power of 400 magnification. Triplicate slides were made for the assay. At least 100-200 cells were evaluated per spot on the slide within the grid of the eyepiece of the microscope from 10 random fields. Two trained researchers were assigned blindly to read and results were averaged. The results are expressed in percentage of acrosome-reacted spermatozoa in the total population counted.

Experiments could also test control sperm in Ham's F-10 / 0.5%HSA versus test sperm exposed to the different cell transfection and protein separation conditions of non-transfected PA-1 cells (NT) as well as transfected rhZP3 expression PA-1 cells, native solubilized zona proteins, chemical agents, oligopeptide of mouse and human ZP3. Recombinant human ZP3 used in the present study came from both Frozen (-196°C liquid nitrogen) and Fresh (protein eluted followed by the assay without frozen).

5. Dose-dependency studies

Two assays were used for this investigation. First, different rhZP3 concentrations were used for testing in order to obtain the optimal concentration

of rhZP3 synthesized for inducing acrosome reaction measured by immunofluorescence techniques outlined in the previous section. Serial dilutions of a wide range of rhZP3 concentration were done first. Then as the broad range was known, concentrations of rhZP3 with closer concentration difference were used. A curve of acrosome reaction vs. concentrations of rhZP3 was drawn from the data to get the optimal concentration. Same method of experimental setup was used for the HZA as previously described. The result was expressed as the HZI that was previously described and a curve of HZI vs. concentrations of rhZP3 could used to obtain the optimal concentration of rhZP3.

6. Time course studies of capacitation

The motility and acrosome status were examined for both control and rhZP3-treated sperm under the basal conditions for spontaneous acrosome reaction by the Hamilton-Throne computerized sperm analyzer and the acrosome reaction assay respectively. Different sperm capacitation time spans (1, 2, 4, 8, and 24 hr) were tested. Analysis of Variance with Bonferroni Multiple-Comparison Test was used for multiple comparisons.

7. Oligopeptides mouse ZP3-1 and human ZP3-6

Mouse ZP3-1 and human ZP3-6 were oligopeptides from the ZP3 of respective species. The amino sequence of ZP3-1 is T-P-S-P-L-P-D-P-N-S-S-P-Y-H-F-I-V-D-F whereas the amino sequence of ZP3-6 is D-V-T-V-G-P-L-I-F-L. The prepared sperm samples were induced for acrosome reaction by mouse ZP3-1, human ZP3-6 and rhZP3. The concentration for the experiment was at

30 ng/mL level. Controls for this experiment were Ham's F-10 (negative), 30 ng/mL non-transfected PA-1 cells (NT) and 5 mM calcium ionophore A23187 (Sigma). All the conditions were the same as previously described.

8. G-protein antangonist: Inhibition of G-protein

This experiment utilized the binding test of hemizona assay. One test and control experiments were prepared using the sperm sample from one single ejaculate. For the test experiment, the concentration-adjusted test sperm droplet was pre-treated with 100 ng/mL pertussis toxin for three hours. Then both the test and control sperm droplets were treated with 500 ng/mL rhZP3 for 30 min. The corresponding hemizona were placed in each droplets for 3 hours. The HZIs were then determined and compared.

9. G-protein antagonist: Involvement of G-protein in acrosome reaction signaling cascade

One test and control experiments were prepared using the sperm sample from one single ejaculation. The test group pretreated the sperm with 100 ng/mL pertussis toxin, an antagonist of G-protein immediately after swim-up followed by the rhZP3 while the control was rhZP3 treatment alone. After the capacitation of the sperm, both treatment and control groups were incubated with rhZP3 for 30 minutes at 95% air, 5% CO_2, 37°C just as the method and condition previously discussed. In other words, both the pertussis toxin-treated sperm and non-pertussis toxin treated droplets are then exposed to the optimal concentration for acrosome reaction of rhZP3. The droplets were subjected to the immunofluoresence staining of acrosome status as described earlier. Then

the results were compare for the pertussis toxin-treated followed by the rhZP3 sperm with its rhZP3-treated droplets.

10. Intracellular Ca^{2+} concentration change for rhZP3-treated and potentiation of rhZP3 with physiological and chemical compound.

Fluorospectroscopy was widely used in biochemistry because of the sensitivity and selectivity the technique provides for the analyst by using a luminescent molecular probe. A major advance in ion indicators and fluorescent tracers has been the development of lipophilic, membrane-permeant esters that cross the cell membrane and are then hydrolyzed by intracellular esterases to yield the active, membrane-impermeant probe molecule inside the cell (Grynkiewicz, et al., 1985). FURA-2 has two excitation wavelengths at 340 nm and 380 nm as well as an emission wavelength at 510 nm. It is this characteristic of the dye that is used to load cells for a typical intracellular calcium measurement (Grynkiewicz, et al., 1985).

The experiments of the present study consisted of monitoring the time course of the 340/380 wavelength ratio measurements. The experiment monitored sperm cells that were exposed to a calcium regulatory agent such as a recombinant protein, a hormone or chemical reagents. The resulting ion transients were then recorded via the 340/380 wavelength ratio because chelated FURA-2 and unbound FURA-2 gave a shift in the excitation (Becker and Fay, 1987). For FURA-2 the 340 wavelength must increase while the 380 wavelength must simultaneously decrease. This was because the 340 wavelength increase was a product of the ion-dye chelation complex; whereas, the 380 wavelength decrease was a result of the free dye decrease (Roe et al.,

1990).

The present study used the facility in Dr. Peter Blackmore's Fluorospecroscopy (Department of Pharmacology. Eastern Virginia Medical School, Norfolk. Virginia).

11. Statistical analysis

The results from test and control groups in the assays were expressed by the descriptive statistics, such as the mean. standard error of mean (SEM), and sample size. A two-tailed paired *t*-test which served the purpose to compare the mean (or median) of a single group to a target value usually a control or baseline will be conducted at a significant level of 0.05. In order to compare one of the treatments to a control group or to compare all of the other means to the mean of this control group (post-test). Bonferroni (Versus Control) Multiple-Comparison Test was used to accomplish the task. The alpha level of the Bonferroni Test is set at 0.05. Bonferroni Test is a variation version of the One-way ANOVA post-test which conducted *t*-tests among the treatments or treatments to the control.

B. Detailed Methods

1. Preparation of semen

a. Swim-up procedure

Semen was obtained from normal donors with consent at Jones Institute for Women's Health (Norfolk, VA) and allowed to liquefy for 30-60 min at room temperature. All semen samples were retrieved in a specimen container (Baxter

Healthcare Corporation, USA). Unless otherwise noted, we used motile sperm selected by the "swim-up" procedure. Swim-up procedure is illustrated as follows. Semen was divided into 0.5-mL aliquots, placed at the bottom of plastic 15 mL tubes. Each aliquot of semen was washed with 2 volumes of Ham's F-10 supplemented with 0.5% human serum albumin (HSA) (Irvine Scientific, Santa Ana, CA). The sperm suspension was centrifuged for 8 minutes at 400 g. The procedure was repeated for a second time. The final pellet was then overlaid with 500 mL of Ham's F-10 / 0.5%HSA and all tubes must be loosely capped before the incubation at a 30^{o} angle for 1 hour at $37^{o}C$ in 5% CO_2; 95% air, allowing sperm to swim from semen into the medium.

b. Setup of Hamilton-Thorn Sperm Analyzer

The parameters for each function of swim-up and semen analysis which was coded as the "Swim-up" and "Basal" in the computer program respectively was used to set up the Hamilton-Thorn Research Sperm Analyzer (Hamilton-Thorn Research, MA) prior to analyze the sperm samples. The parameters that were used at Jones Institute for Women's Health for the present study were listed as follows.

"BASAL"
Apply Sort: 0
Frames Acquired: 30
Frame Rate: 60 Hz
Minimum Contrast: 85
Minimum Cell Size: 4 Pixels
Straightness (STR), Threshold: 80.0 %
Low VAP Cutoff: 5.0 pm/s
Medium VAP Cutoff: 25.0 pm/s
Head Size, Non-Motile: 12 Pixels

Head Intensity, Non-Motile: 130
Static Head Size: 0.25 to 2.98
Static Head Intensity: 0.60 to 1.55
Static Elonqation: 0 to 100
Adaptive Filter:YES
Slow Cells Motile:NO

"SWIM-UP"
Apply Sort: 0
Frames Acquired: 30
Frame Rate: 60 Hz
Minimum Contrast: 80
Minimum Cell Size: 5 Pixels
Straightness (STR), Threshold: 80.0 %
Low VAP Cutoff: 5.0 pm/s
Medium VAP Cutoff: 25.0 pm/s
Head Size, Non-Motile: 5 Pixels
Head Intensity, Non-Motile: 90
Static Head Size: 0.28 to 2.99
Static Head Intensity: 0.55 to 2.40
Static Elonqation: 8 to 100
Adaptive Filter:YES
Slow Cells Motile:YES

c. Sperm Motion with Hamilton-Thorn Sperm Analyzer

Makler counting chamber was used to load 5 μL of the sperm sample into a four-chamber slide and then place the four-chamber slide on the stage of the analyzer. Then after the Hamilton-Thorn Sperm Analyzer was prewarmed to 37°C, "SETUP" and "CONFIGURE STAGE" functions were checked and should have read "Basal" and "Makler", respectively. At this time, the information of the sperm was typed in the "INFO" page: Sample:Diluent, Volume (mL), Semen sample, ID# (donor number) and Collection time. The button labeled "ACQUIRE" was checked to view the loaded sperm sample on the screen. Manipulation could be done by pressing the "JOG OUT" button as many times as required. Then the desired field could be focused by using the large round

focus knob right next to the "JOG IN" button. The functions "START SCAN" and "ADD SCAN", if necessary, were selected for analysis. After the analysis, the "DISPLAY" page was printed.

2. Hemizona Assay.

a. Criteria for donors used in the hemizona assay

The criteria for donors used in the laboratory are:

1. The person must have fathered a child within the preceding two years.
2. Semen count must meet the following limits: motility >50%, total count >60 X 10 /mL (60 million/mL).

Sample must effect > 15 tightly bound sperm to the control hemizona. A good working range for binding of control samples is 15 - 100 sperm per hemizona.

b. Ham's F-10 Medium / 0.5% Human Serum Albumin

The medium used for present study of the HZA is Ham's F- 10 which was obtained on a as needed basis from the IVF Laboratory at the Jones Institute for Women's Health. The medium had been quality control tested using the mouse two-cell assay. Also the medium had been used for one week in the IVF laboratory thereby providing human IVF quality control. The shelf life for the medium is two months in 4°C.

Ham's F-10 medium was supplemented with 0.5% human serum albumin (Irvine Scientific, Ca). The human serum albumin had also been quality control tested by the mouse two-cell assay prior to its use in the laboratory. Under a biological safety hood, 25 mL of IVF Ham's F-10 medium was removed. Then it

was aliquoted into a 50 mL culture flask. In order to obtain a final concentration of 0.5% human serum albumin, HSA (Irvine Scientific, CA). This is achieved by adding 1.25 mL of HSA to Ham's F-10 medium. They were mixed well gently. One day before the experiment was set up, sufficient Ham's F-10 was delivered into the centrifuge tubes and were placed in the 95% air, 5% CO_2, 37°C incubator with caps loosened. Remaining Ham's F-10 with 0.5% HSA can be stored in the refrigerator for one week.

c. Sperm preparation: Swim-up and its adjustment

The pH of Hams F-10 media with 0.5% HSA was equilibrated by placing two 15-mL centrifuge tubes containing the media overnight in the incubator at 37°C with 5% CO_2 in air. Liquefaction of donor semen could be achieved by rocking the specimen container back and forth. At this point sample should flow freely. Use Hamilton-Thorn Sperm Analyzer for motion analysis. The procedure could be found in the previous section "Setup of Hamilton-Thorn Sperm Analyzer", and "Sperm motion analysis with the Hamilton-Thorn Sperm Analyzer" The volume of semen was measured by a serological pipette. Then 1 mL of semen sample was transferred to a 15 mL centrifuge tube. With the addition of 2 mL of Ham's F10 media to each tube, the resulting sperm samples were vortexed gently. Addition tubes were set up for extra volume of the sperm sample. Again all samples were centrifuged for 8 min at 400 x g. Carefully the supernatant was removed using a 9" Pasteur pipette. To each tube, 1 mL of Ham's F-10 media was pipetted and vortex until pellets were broken up. The washing step was repeated one more time. With extra caution, the sperm

pallets were overlaid with Ham's F-10 media. A pellet approximately 5mm in diameter, should be overlaid with 400 μL of Ham's F-10 / 0.5% HSA media. For a sample approximately ≤ 5mm in diameter, it was advised to be overlaid with 150 - 300 μL of Ham's F-10 / 0.5% HSA media. Loosely all tubes was capped and incubated at 37°C with 5% CO_2 in air for 1 hour. Swim-up (SU) samples were measured for their motion analysis on the Hamilton-Thorn Analyzer after an hour. This would be a determination of the dilution to make for the hemacytometer assessment of sperm concentration. The "final" dilution of motile sperm concentration for the HZA in the present study was 0.5×10^6 motile sperm /mL.

d. Hemizonae setup

Salt-stored nonviable human oocytes obtained from the IVF laboratory at Jones Institute for Women's Health were bisected by micromanipulation prior to their use in the assay. An inverted, phase-contrast microscope (Nikon Diaphot. Garden City, NJ) was equipped with a micromanipulation system (Narishige Model MO-102, Tokyo, Japan). The micromanipulator was also equipped with a Beaver Micro-sharp blade (Beaver, #7530) attached. Beaver handles and blades were obtained from Surgical Specialist in Crofton, MD. The cutting chamber was the top half of a 100 x 15 mm petri dish (Falcon #7530) which was secured to the stage with tape. The oocyte-rinsing medium contained Ham's F-10 / 0.5 % BSA (bovine serum albumin). A pulled capillary tube (micropipette) was made and scored to break off at approximately 1.5 times oocytes diameter to the pipetter bulb. After removing the oocyte from salt storage solution, a drop

of Ham's F-10/0.1% BSA was placed in a 100 X 15 mm petri dish. The oocytes was washed thoroughly with the micropipette. The knife/handle system should be set up so that the knife handle was at a 45 angle to the stage and its cutting surface was perpendicular to the cutting dish.

Straight positioning of the knife body was checked by looking at the shadows casted by the blade on the dish. If the shadows on both sides of the blade were equal. the blade was properly positioned. Adjustments were made until these criteria were met. Some Ham's F-10 / 0.5% BSA prepared previously was laid on cutting area. Then three parallel grooves were created on the bottom of the dish with knife.

A salt-stored oocyte was washed 5 times by micropipette in 5 drops of Ham's F-10 / 0.5% HSA . Then this washed oocyte was transferred to one of the cutting lines for resistance to rolling. Slowly the blade was lowered over the middle of the oocyte to make the bisection. Both the matching hemizonae were removed from the droplet of medium. Next was to transfer the hemizona to a culture dish. The matching hemizonae were visualized with a dissecting microscope. Any ooplasm from each hemizonae should be removed with the micropipette as described above for rinsing of oocytes. Hemizonae were placed in a single drop of salt storage solution under heavy mineral oil. Hemizonae can be stored at 4°C until 24 hours prior to use in the hemizona assay.

e. Hemizona assay

After the donor swim-up, the hemizona assay was proceeded. The sperm concentration should be adjusted to 0.5 X 10^6. Droplets of 100 μL

amount of diluted donor sperm was placed in the control area in a petri dish. Another 100 μl droplet of donor diluted sperm was treated with appropriate rhZP3 concentration (30 ng/mL or 500 ng/mL), or pertussis-toxin (100 ng/mL) or Non-transfected (30 ng/mL or 500 ng/mL). One hemizona was placed on the control droplet and its matching hemizona was placed on the corresponding treated sperm droplet. Each experiment should have three pairs of hemizonae used to claim its validity. All dishes were incubated at 37°C. 5% CO_2 in air for 4 hours.

f. Reading of hemizona assay

The number of sperm bound to the outer surface of each hemizona was determined after the four-hour coincubation. Counting dishes (60 mm petri dishes) was labeled with the abbreviation, T, for treatment on one half of the dish for treatment and the abbreviation, C, for control on the other half of dish. Ham's F-10 / 0.5% BSA droplet was placed on the corresponding column.

The dish containing the hemizonae was taken out for evaluation from the incubator. It was crucial to remove only one hemizona one at a time to be rinsed 15 times for 3 separate drops at salt solution. Then each hemizona was transferred to the corresponding spot in the counting dish. This step was repeated until all hemizonae were washed. Effort was made to put the hemizonae in the correct labeled column. Then the counting dish was placed in a refrigerator for 30 min. After then the counting of sperm on the hemizonae was proceeded. The hemizona was manipulated so that the cut surface was down against the counting dish. All the planes were focused up through the

hemizona surface counting all sperm bound on the outer surface of the hemizona. The counting process started at 12:00 and proceeded clockwise around the circumference of the zonae. Sperm tails extended outward were counted. The zonae was then positioned on its side and sperm tails on the outer surface of the zonae were counted. Finally, the zonae was manipulated to have its outside bound sperm count. Caution was made not to count sperm bound to the inner surface of the zonae. The hemizona index (HZI) was calculated as follows:

$$\text{Hemizona Index (HZI)} = \frac{\text{\# of bound test sperm}}{\text{\# of bound control sperm}} \times 100$$

The HZI is expressed as a number, not as a percentage.

g. Dose-dependency studies

A total of 3 donors meeting the criteria listed in the previous section were used to test 2 different lots of rhZP3 purification of exact procedure. The tested concentrations were 10, 30, 100, 250, 500, and 2000 ng/mL rhZP3. Positive control (calcium ionophore) and negative controls (Ham's F-10 and non-transfected PA-1, NT) were used. The entire experimental set-up and conditions were done exactly like the section "Hemizona Assay." The only addition was the variable concentrations (doses) of rhZP3 from 2 different lots of purification of the same procedure.

h. Binding test

Semen obtained were processed for swim-up as the section "Sperm

preparation: Swim-up and its adjustment ." The sperm concentration for the present assay was adjusted to 0.5 million/mL. The testing concentrations that we used for the inhibition binding assay were 30 ng/mL and 500 ng/mL. A total of 3 donor sperm were used and each experiment utilized 3 matching hemizonae. A paired series of 100μl of sperm droplets were prepared for rhZP3-treated and Ham's F-10 (negative control) for each pair of hemizonae. Some swimup sperm were saved for the immunofluorescence detection of acrosome reaction. Add appropriate dilution of the concentration of rhZP3 to achieve a final concentration of 30 or 500 ng/mL of rhZP3 to the droplet. The rhZP3-treated sperm droplets were incubated for 0.5 hour prior to the placement of matching hemizonae to its corresponding droplets, rhZP3-treated and negative control. The incubation was in 95% air, 5% CO_2, 37°C incubator for 3 hours. At the end of three hours, remove only one hemizona one at a time to be rinsed 15 times for 3 separate drops at salt solution. Then each hemizona was transferred to the corresponding spot in the counting dish. This step was repeated until all hemizonae were washed. The score of HZI was read as described in the section "Reading of hemizona assay."

3. Immunofluorescence techniques of acrosome reaction

a. Sperm preparation

Sperm samples were from donor pool that were complied with the donor selection criteria listed in "Criteria for donors used in the hemizona assay." Then process the sperm semen as in the section "Sperm preparation: Swim-up and its adjustment." The sperm concentration for the immunofluorescence detection of

acrosome reaction was set at 2 million/mL.

b. Stock solution for Calcium ionophore A23187

In 3.8 mL of DMSO (Sigma. St. Louis. MO), 10 mg calcium ionophore A23187 (Sigma) was dissolved to give a concentration of 500 mM. Then 50 μL of the ionophore solution was aliquoted into sterile containers (Eppendorf) tubes. For each 50 μL aliquot, 450 μL of DMSO (Sigma) was added. The final stock solution was aliquoted with 40 μL into each 0.5 μL Eppendorf tube. Aliquots might be stored at room temperature for up to 6 months.

c. Working solution for Calcium ionophore

A working solution of 100 mM Calcium ionophore A23187 solution was prepared by adding 40 μL Ham's F-10 (or appropriate capacitation media) to 10 μL of 500 μM calcium ionophore A23187 stock solution. (This gave a 100 μM calcium ionophore A23187 solution.)

d. Preparation of testing-, positive control-, and negative-control sperm suspensions

A series of 100 μl sperm aliquots (positive control of calcium ionophore. negative, and test) was prepared according to the concentration desired. The final preparation was placed in the Eppendorf vials and all vials were incubated for 30 minutes the 95% air, 5% CO_2, 37°C incubator. For the positive control, 5 μL of the 100 μM calcium ionophore A23187 (Sigma) solution was delivered to 95 μL of the sperm suspension and let it incubate for 30 minutes the 95% air, 5% CO_2, 37°C incubator. The final concentration of the capacitation media

would then be 5 μM of calcium ionophore A23187 (Sigma).

e. Preparation of spot slides

Triplicate slides were be made for the assay. An amount of 300 μl PBS was introduced into each positive, negative, and test vials which were mixed by gentle vortex. Then all Eppendorf tubes were centrifuged at 3000 rpm or 400 x g for 8 min. After that the supernatants from each tube were discarded. pallets in the bottom of the Eppendorf tube was resuspended in 90 μl Ham's F-10 (or PBS). Hoechst 33258 stain was prepared as follows. Each vial was added 10 μl Hoechst 33258 stain in 990 μl Ham's F-10 or PBS. Then to each vial 10 μL diluted Hoechst 33258 stain solution was introduced and all tubes were incubated in 37°C at 5% CO_2 for 15 min. Upon completion of incubation, all vials were washed in Ham's F-10 (or PBS) at 3000 rpm or 400 x g for 8 min. Then the supernatant from each tube was discarded. At this point the sperm fixative was prepared by mixing 10 μl azide (Sigma, St. Louis, MO), 10 μl PMSF (Sigma, St. Louis, MO) and 80 μl Ham's. An amount of 10 μl of the sperm fixative in each Eppendorf vial was added. The pallets were immediately washed in 1 mL PBS at 3000 rpm or 400 x g for 8 min. Two more washes were proceeded after that. Pallets from the final wash were resuspended in 60 μl PBS. A labeled slide was spotted with 10 μl sperm suspension from the corresponding vial onto the spots. The slides were air dried for two hours or overnight in a dark box. Next, the spot slides were immersed in methanol for 10 min followed by PBS for 10 min for a total of two times. The washed slides were air dried in the dark and were stored in a light-seal box in -80°C until use.

f. FITC/PSA staining

A paper towel was moistened with PBS and placed in bottom of slide box. FITC/PSA 1:20 (10 µl FITC/PSA + 190 µl PBS) was prepared in an Eppendorf tube. The slide spots were covered with 25 µl PBS for 10-15 minutes prior to staining. Then with kimwipes the PBS was removed. An amount of 25 µl of the FITC/PSA was distributed to each spot. The slides were incubated in a closed slide box in the dark for 1 hr. At this time, the fluorescent scope (at least 30 min prior to use) was turned on. After 1 hour of incubation, FITC/PSA was removed from all spots with kimwipes. Each spot was rinsed 3 times,10 minutes each time, with PBS. Then with kimwipes PBS from each spot was removed. Antiquench, stored at –80°C, was added with an amount of 5 µl was onto each spot. The slides were covered with coverslips. The slides should be placed in the darkness until time to be read. Slides can be stored in the dark at -20°C for 2 weeks.

g. Slide Reading

At least 100-200 cells was evaluated per spot slide within the grid of the eyepiece of the Nikon fluorescent microscope (Tokyo, Japan) from 10 random fields. Two trained researchers were assigned to read and the results averaged. The result was expressed in percentile of acrosome-reacted spermatozoa in the total population counted. The state of acrosome was scored in accordance to the four categories that were identified by Mortimer et al. (1989). I, "intact" demonstrated an even fluorescence; II, "patchy" where at least 20% of the

acrosome cap region showed no fluorescence; III "Equatorial segment" where only the equatorial segment of the acrosome showed fluorescence; and IV. "dark" where there was no fluorescence labeling visible on the sperm head.

h. Solubilization of zona pellucida

Ooplasma was removed using a small bore glass micropipette. A vigorous pipetting action would break their zona causing ooplasma to be spilled into surrounding medium. The chosen amount of zonae was transferred into an Eppendorf tube and was centrifuge for 15 minutes at 300 x g. Using a stereomicroscope, the media were removed with pipette, making sure not to remove disturb zona. The final result was to remove maximum medium leaving zonae only in a film of medium. A chosen volume (depending on the zona concentration needed) of 10 mM HCl was added. Under the microscopic vision. zonae/HCl was pipetted up and down until all zonae were dissolved. Then 10 mM NaOH of equal volume as the 10mM HCl was added and mixed well. This was regarded as a STOCK zonae solution.

i. Dose-dependency studies

Four ejaculatory semen samples meeting the criteria selection as indicated in the previous section "Criteria for donors used in the hemizona assay" were used for the sample pool. The process of experiment was same to the sections a through g of "Immunofluorescence techniques of acrosome reaction." The only variables were the concentrations of rhZP3 which were 7.5. 15, 30, 60, 120, 240, 480, 960, and 1920 ng/mL rhZP3. The readings were

scored for Hoescht's and FITC-PSA staining.

j. Acrosome reaction: Immunofluorescence techniques

Twenty-nine semen samples from 11 donors who met the criteria selection as indicated in the previous section "Criteria for donors used in the hemizona assay" were used for the sample pool. The procedure was done exactly like the sections a through g of "Immunofluorescence techniques of acrosome reaction." The concentration of rhZP3 in the study was 30 ng/mL. The study also looked at 500 ng/mL rhZP3 and 0.5ZP/mL of solubilized zona was prepared from the stock solution of solubilized zona described previously in "Solubilization of zona pellucida." Appropriate positive- and negative controls were used as previously described.

k. Transmission Electron Microscopy

(1) Sperm preparation

Healthy donors provided fresh semen after they had given informed consent. Completely liquefied ejaculates were analyzed for the standard semen parameters according to the World Health Organization (WHO), 1992. The sperm samples are subjected to the swimup and sperm motility analysis procedure followed by 4-hr capacitation in 5% CO_2 incubation at 37°C. After the second determination of motility, the sperm concentration was adjusted to 4.5-9.0 X 10^6. The sperm samples of 0.5 mL aliquots were pipetted into 1.5 mL Eppendorf tubes.

(2) Induction of acrosome reaction

The sperm aliquots were subjected to four different treatments namely negative, calcium ionophore A23187, recombinant human ZP3, and solubilized zona. Stock solutions of calcium ionophore A23187 (500 μM in 100% DMSO) were stored at -20°C. Working solutions were prepared by diluting the thawed stock solutions of ionophore 1:5 or 10 μL calcium ionophore A23187 in 40 μL of Human Tubal Fluid or Ham F-10 medium (without Human Serum Albumin or HSA) 30 minutes before stimulation of the acrosome reaction. The recombinant human ZP3 was prepared from its stock concentration to 500 ng/mL in the Human Tubal Fluid or Ham F-10 medium (supplemented with 0.5% HSA). Treat the 100 mL sperm aliquot with various treatments and incubate at 37°C, 5% CO_2 in air for 30 min. Then proceed to two times washing in 300 mL PBS at 400x g for 8 min.

(3) Immunofluorescent detection of acrosome reaction

The procedure was similar to the "Preparation of testing, positive control, and negative-control sperm suspension" and "Preparation of spot slides." The rhZP3 concentration of this experiment was 500 ng/mL. Appropriate positive and negative controls were included as described in previous section.

(4) Transmission electron microscopy

Spermatozoa were fixed by mixing sperm suspensions with equal volumes of 2% (v/v) glutaraldehyde (in 3 mM $CaCl_2$ and 0.1 M sodium cacodylate). After 24 h at 4°C, the sperm was washed three times (10 min) in

0.1 M sodium cacodylate, and postfixed for 90 min in 1% (w/v) uranyl acetate for 90 min (dark chamber). Thereafter, the samples were dehydrated twice in 70% (v/v), 95% (v/v), 100% ethanol, and 100% (v/v) propylene for 10 min each. The fixed and dehydrated sperm pellets were embedded in a modified plastic [(12 g Araldite 506 (Electron Microscopy Sciences. Washington, PA), 13 g Poly/Bed (Polyscience, Warrington, PA), 25 g dodecenyl succinic anhydride (Electron Microscopy Sciences), 0.9 mL of 2.4,6,-tri(dimethylaminomethyl)-phenol (Electron Microscopy Sciences)] and incubate in the oven at 68°C for 48 hours (Kohn et. al., 1997).

After thin sectioning with diamond knife and staining in 2.5% (w/v) uranyl acetate (in 50% ethanol) and lead (80.3 mM lead nitrate, 119.7 mM sodium citrate in deionized water, pH 12.0), 72-141 spermatozoa per sample (controls and treated samples) were examined by means of a transmission electron microscope. The morphology of all acrosomal status of spermatozoa was examined by a Transmission Electron Microscope (Jeol JEM-1200 EX II. Peabody, MA).

4. Oligopeptide mouse ZP3-1 and human ZP3-6

The sperm sample was prepared and read according to the procedure a through g in "Immunofluorescence techniques of acrosome reaction" procedures described previously. Then apply 30 ng/mL of mouse ZP3-1 and human ZP3-6 respectively to the sperm droplets. The rhZP3 was also included for comparison at 30 ng/mL level. The controls for this experiment were Ham's F-10 (negative), 30 ng/mL non-transfected PA-1 cells (NT) and 5 μM calcium ionophore A23187

(Sigma). All the conditions for the experimental procedures were the same as previously described in "Chapter III, Section B, 3. Immunofluorescence techniques of acrosome reaction."

5. G-protein

The sperm sample was prepared by sections a through g of "Immunofluorescence techniques of acrosome reaction" procedures as described previously. After the swim-up, the sperm in the treated vial was treated with100 ng/mL of pertussis toxin and 500 ng/mL rhZP3 whereas the control sperm was treated 500 ng/mL rhZP3 only. The incubation time for the pertussis toxin in the test experiment was 4 hours, elapsing the entire capacitation time. All conditions are the same as previously described.

The other test for the G-protein antagonist utilized the hemizona assay. The conditions are the same as Chapter III, Section B, 1. "Preparation of Semen" and 2. "Hemizona Assay" unless otherwise stated. The variation of the conditions were 100 μL sperm droplets were divided into test and control: the test sperm droplet was 1 μL of 100 ng/mL pertussis toxin-treated and the control sperm droplet was 1 μL Ham's F-10 / 0.5% HSA . Both were incubated 37°C. 5% CO_2 in air for 3 hours. Then 1 μL of an adjusted amount of rhZP3 was added to attain a final concentration of 500 ng/ml of rhZP3 in the droplet. The incubation time for the 500 ng/ml rhZP3 was 30 min at 37°C, 5% CO_2 in air. The pre-washed hemizonae were introduced into its corresponding droplets and were incubated at 37°C, 5% CO_2 in air for 4 hours. Immediately, the hemizonae were washed and counted for HZI by the method that was described previously.

6. Fura-2 assay

Fura 2 assay for measurement of $[Ca^{2+}]_i$ is performed at Dr. Peter Blackmore's laboratory Dr. Peter Blackmore did the measurement himself because of the complexity and delicacy of the instrument. Preparation of human sperm was similar to the procedure previously described in Immunofluorescent . following overnight capacitation or noncapacitated. were loaded with fura 2 essentially as previously described (Thomas and Meizel, 1988). Sperm (5 to10 x 10^6) were incubated with 4mM fura 2-AM for 45 min at 37°C. After centrifugation (2000g for 5 min) cells were resuspended in FM3B buffer then kept in the dark at room temperature to prevent photobleaching. Aliquots (0.5 mL) of sperms were incubated at 37°C in 6 x 50-mm glass test tubes containing a small magnetic stirring bar in a SPEX ARMC spectrofluorometer. Aliquots of rhZP3 were then added to the sperm suspension 30 s after data collection was started. Sperm were excited at 340 and 380 nm, respectively, and emission measured at 505 nm. Data was collected between 2 and 5 min depending on the protocol used. The integration time was 0.1 s with a time increment of 0.5 s. On completion of experiment, sperm were lysed with 0.01% (w/v) digitonin, then 10 mM EGTA was added to obtain fluorescence values of fura-2 at both wavelengths when it was either saturated or depleted of calcium. Autofluorescence of the cells was determined at both wavelengths by adding 2 mM $MnCl_2$, in the presence of 20 mM ionomycin to fura 2-loaded cells. The autofluorescence values were then subtracted from the values obtained in the fura 2-loaded cells and the levels of $[Ca^{2+}]_i$ calculated.

CHAPTER IV

RESULTS

A. *In vitro* expression, isolation, and purification of recombinant human Zona Pellucida 3

One of the goals of the present study is to assist in seeking the optimal purification conditions of the rhZP3 in our laboratory. The process of trial and error was lengthy but the end result was rewarding. The final expression, isolation, and purification conditions procedure are listed in the section of Experimental Design. And it was reported that the WGA / Ni-NTA affinity chromatography combination can purify the rhZP3 glycoprotein at 60% purity. In addition, the purified rhZP3 has the M.W. = 62 ~66 kDa, pI = 3.4 ~5.0 (Chi. 1998).

B. Hemizona assay (HZA): Dose-dependency studies

In order to determine how the concentrations of rhZP3 affected the inhibition of binding of sperm to zonae, a dose-dependency assay was set up for 10, 30, 100, 250, 500, and 2000 ng/mL rhZP3. Table 1 is a dose-dependency study of rhZP3 using the Hemizona assay. The total number of donors used was 3 and the number of rhZP3 purification lots used were 2 for the present study. Also each experiment for the Hemizona was analyzed using three matching hemizonae to claim its validity. The concentration of the sperm in the test was set at 0.5 million per mL. The Mean ± SEM and their *p*-values were included in Table 1. Figure 1, which illustrates the HZI versus its treatment

concentration of rhZP3. depicts a plot of the data in Table 1. In Figure 1. the hyperbola curve starts decrease dramatically during the first 30 ng/mL of rhZP3 (from HZI 92.9 ±1.6 to 61.5 ± 2.8) then the rate of decreasing retarded followed by a level-off at around 500 ng/mL of rhZP3 at HZI of 36.6 ± 1.4.

C. Hemizona assay (HZA): Sperm-Zona Binding Test

Table 2 shows the Hemizona index (HZI) obtained from the Hemizona assays performed for 30 ng/mL and 500 ng/mL rhZP3-treated, Ham's F-10. as well as non-transfected PA-1 against its corresponding controls of Ham's F-10 / 0.5%HSA, 30 ng/mL NT, and 500 ng/mL NT. Each experiment was done with triplicate matching pairs of hemizonae for a total of 3 donors in a sperm concentration of 0.5 million per mL. In Table 2, the pair *t*-test indicates the HZI for 30 ng/mL rhZP3 vs. Ham F-10 / 0.5% HSA (43.6 ±3.3) and 30 ng/mL rhZP3 vs. 30 ng/mL NT (44.5±3.6) is significantly different ($p<0.01$) from 30 ng/mL NT vs. Ham's F-10 / 0.5%HSA (94.2 ± 0.3). Also, 500 ng/mL rhZP3 vs. Ham F-10 / 0.5% HSA (38.0 ± 2.7) and 500 ng/mL rhZP3 vs. 500 ng/mL NT (41.8 ± 1.9) were significantly different ($p<0.01$) from 500 ng/mL NT vs. Ham F-10 / 0.5% HSA (93.4 ± 0.1). Table 3 indicates the percentage of acrosome-reacted sperm in the droplets that were used for the inhibition binding assay of the hemizona. The data indicates the percentage of acrosome reaction of rhZP3-treated sperm which was previously exposed to the protein for 0.5 hour after swim-up, Ham's F-10 and non-transfected PA-1 (NT) collected and processed at the time frame immediately after the swim-up and at the end of 3-hour incubation.

Table 1. Dose Dependency Study of rhZP3. A total of 3 donors and two different lots of rhZP3 were used for the assay. Each concentration of rhZP3 was tested with 3 matching pairs of hemizonae.

Conc. of rhZP3 in ng/mL	HZI Mean (±SEM)
2000	35.8 (0.9)*
500	36.6 (1.4)*
250	45.5 (2.4)*
100	55.4 (3.3)*
30	61.5 (2.8)*
10	92.9 (1.6)

n = 3 donors x 3 pairs of matching hemizonae per concentration testing
2 lots of rhZP3
*$p<0.01$

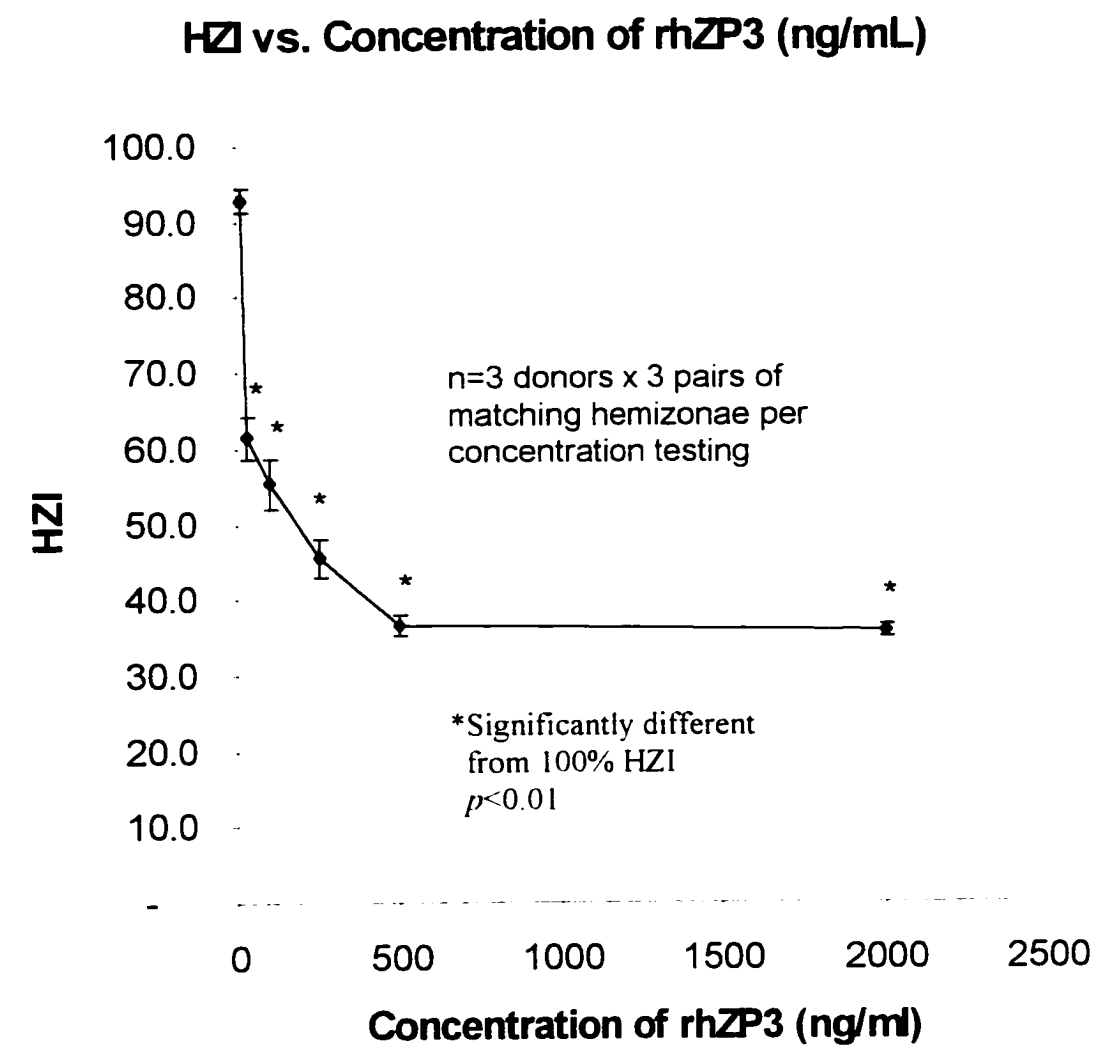

Figure 1. HZI Vs. Concentration of rhZP3 (ng/mL). A plot of the data in Table 1 for HZI versus its corresponding rhZP3 concentration in ng/mL.

and was compared against the Ham's F-10 sperm or non-transfected PA-1 (NT) droplet that had no exposure to rhZP3. Then the matching hemizonae were loaded to co-incubate with the droplets for 3 hours. Table 3 was a control study of the binding test which illustrated that the acrosome-reacted sperm from rhZP3-treated hemizona droplets after 3 hours of incubation at 37°C, 5%CO_2, 95%air were not statistically significant from negative droplets. In other words, the acrosome-reacted sperm induced by the rhZP3 (HZI 14.8 ± 2.7) in sperm droplets, although they may contribute to a lesser degree, was not significant (HZI 9.6 ± 1.1 for the negative control and HZI 10.4 ± 2.3 for the non-transfected PA-1) to cause the inhibition of the binding of the sperm to the hemizona.

D. Dose-dependency studies of acrosome reaction

The present study also analyzed the dose-dependency of the rhZP3. Table 4A shows the mean ± SEM percentage of acrosome-reacted spermatozoa from a total of four donors. Statistical analysis using Bonferroni (Versus Control) Multiple-Comparison Test for dose-dependency study of rhZP3 at a concentration level in 0, 7.5, 15, 30, 60, 120, 240, 480, 960, and 1920 ng/mL was performed to see whether there was any significance among concentration groups. The results indicated that the concentrations of 30, 60, 120, 240, 480, 960 and 1920 ng/mL were significant at the $p<0.05$. In other words, the dose-dependency study of rhZP3 showed that the concentration of rhZP3 will be assert an effective dosage of 30 ng/mL and above on the acrosome reaction of the spermatozoa increased from the baseline acrosome reaction. In addition Table 4B listed the concentration groups that were significantly different

Table 2. HZI at 30 and 500 ng/mL rhZP3. Hemizona index (HZI) of 30 ng/mL and 500 ng/mL rhZP3-treated, and non-transfected PA-1 (NT), with its corresponding controls, Ham's F-10 / 0.5%HSA, 30 ng/mL NT and 500 ng/mL NT. Three donors were used in the experiment. Each experiment was tested with 3 matching pairs of hemizonae.

n=3 donors x 3 pairs of matching hemizonae

Test Reagents	Controls	HZI
30 ng/mL rhZP3	Ham's F-10/0.5% HSA	43.6 (3.3)[a]
30 ng/mL rhZP3	30 ng/mL NT	44.5 (3.6)[a]
30 ng/mL NT	Ham's F-10/0.5% HSA	94.2 (0.3)
500 ng/mL rhZP3	Ham's F-10/0.5% HSA	38.0 (2.7)[b]
500 ng/mL rhZP3	500 ng/mL NT	41.8 (1.9)[b]
500 ng/mL NT	Ham's F-10/0.5% HSA	93.4 (1.0)

Mean (±SEM)

[a] $P<0.01$ as compare to 30ng/mL NT vs. Ham's F-10 / 0.5% HSA
[b] $P<0.01$ as compare to 500 ng/mL NT vs. Ham's F-10 / 0.5% HSA

Table 3. Acrosome-Reacted Status of Control Study. Percentage of acrosome-reacted sperm in the droplets that were used for the inhibition binding assay of the hemizona. The data indicates the percentage of acrosome reaction of rhZP3-treated sperm, Ham's F-10 and non-transfected PA-1 (NT) immediately after swim-up and at the end of 3 hour incubation. The rhZP3-treated sperm droplet was previously exposed to the protein for 0.5 hour after swim-up and was compared against the Ham's F-10 sperm or non-transfected PA-1 (NT) droplet that had no exposure to rhZP3. Then the matching hemizonae were loaded to co-incubate with the droplets for 3 hours. The HZI obtained was indicated in Table 2 and the corresponding percentages of acrosome reaction of each test are listed here (Mean ±SEM)

n=2

%AR after swim-up	%AR of negative 3 hrs. after binding	%AR of NT 3 hrs after binding	%AR of rhZP3 3 hrs after binding
3.2 (0.2)	9.6 (1.1)	10.4 (2.3)	14.8 (2.7)

Table 4. (A) Dose-Dependency Study of rhZP3 using Immunofluoresence Technique. The sperm samples from four donors were treated with different concentrations of rhZP3 (0,7.5,15,30,60,120,240,480,960. and 1920 ng/mL). The percentage of acrosome-reacted spermatozoa are expressed in mean ± SEM.

n=4

RhZP3 Concentration in Ng/mL	% Acrosome-reacted sperm (Mean)	±SEM
0	8.93%	2.17%
7.5	9.01%	2.00%
15	9.87%	2.53%
30	15.22%*	3.76%
60	18.10% *	2.67%
120	19.53%*	2.70%
240	20.55% *	2.43%
480	22.95%*	4.12%
960	24.49% *	5.15%
1920	25.03%*	5.34%

$P<0.05$

(B) Bonferroni (Versus Control) Multiple-Comparison Test for dose-dependency study of rhZP3 at a concentration level in 0,7.5,15,30,60,120,240,480,960. and 1920 ng/mL in Table 4A. Groups that are significantly different at an alpha = 0.05 from the others are listed. The data is based on Table 7A.

Response concentration groups: 0,7.5,15,30,60,120,240,480,960,1920
n=4 Alpha=0.050 DF=30 MSE=1.223656E-03 Critical Value=2.987961

Concentration of rhZP3 in ng/mL	Different from the concentration Groups
0	60, 120, 240, 480, 960, 1920
7.5	60, 120, 240, 480, 960, 1920
15	60, 120, 240, 480, 960, 1920
30	480, 960, 1920
60	0, 7.5, 15
120	0, 7.5, 15
240	0, 7.5, 15
480	0, 7.5, 15, 30
960	0, 7.5, 15, 30
1920	0, 7.5, 15, 30

at an alpha = 0.05 from the others. The concentration groups of 0. 7.5. 15 and 30 ng/mL did not have significant difference among them. However. the concentration groups 0. 7.5, and 15 ng/mL were different at the alpha = 0.05 level from that of

60. 120. 240. 480. 960. and 1920 ng/mL which did not achieve statistical difference among themselves. Figure 2 was a direct plot of dose-dependency study of rhZP3. Note that the curve started to level off at around 500 ng/mL of rhZP3. And a modified version of graphical analysis adopted from Lineweaver-Burk plot to determine the maximum percentage of acrosome-reacted sperm was depicted in Figure 3. Since 1 / % acrosome-reacted sperm versus 1 / rhZP3 concentration in ng/mL gave a straight line. the equation Y=mX+C with m was the slope and C was the y interception of straight line. By computing the reciprocal of the interception. which gave a 24.9% of acrosome-reacted sperm possibly for our rhZP3. R^2=0.91 gave a significant correlation of straight line between the values at Y and X-axis.

E. Immunofluorescence detection of sperm in response to rhZP3

Table 5 shows the percentage of acrosome-reacted sperm as detected in the Immunofluorescence techniques of acrosome reaction in response to 30 ng/mL of rhZP3 from 3 successful purifications using the same method and purification conditions. In the present study, twenty-nine semen samples prepared from 11 donors, who were complied with the donor criterion, as described in IIIB Section 2a entitled "Criteria for donors used in the hemizona assay", employed at Jones Institute for Reproductive Medicine, were

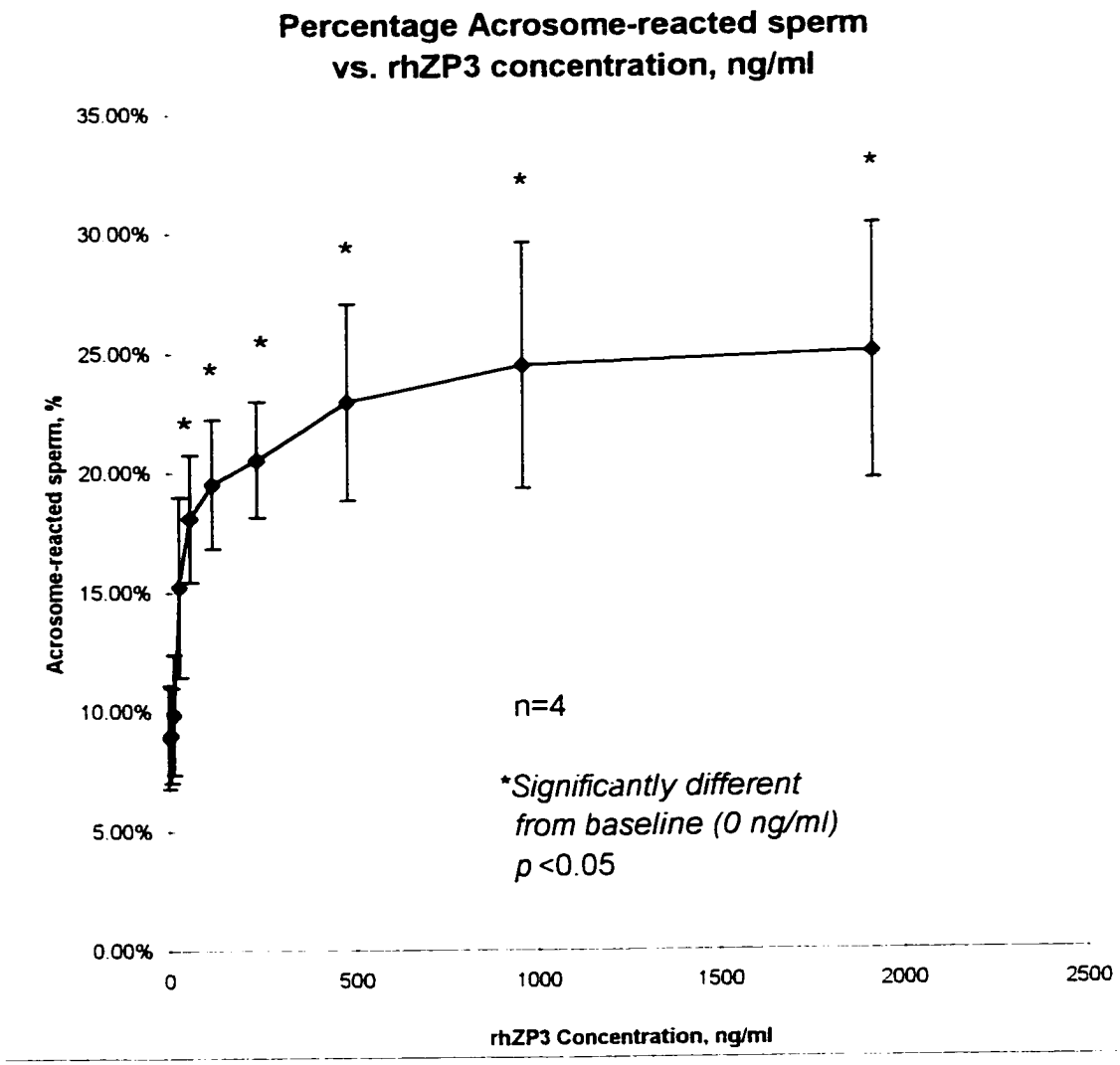

Figure 2. Dose-Dependency Study of rhZP3. Dose-dependency study of rhZP3 of percentage of acrosome-reacted spermatozoa versus the rhZP3 concentration in ng/mL. The plot utilizes the data in Table 7A.

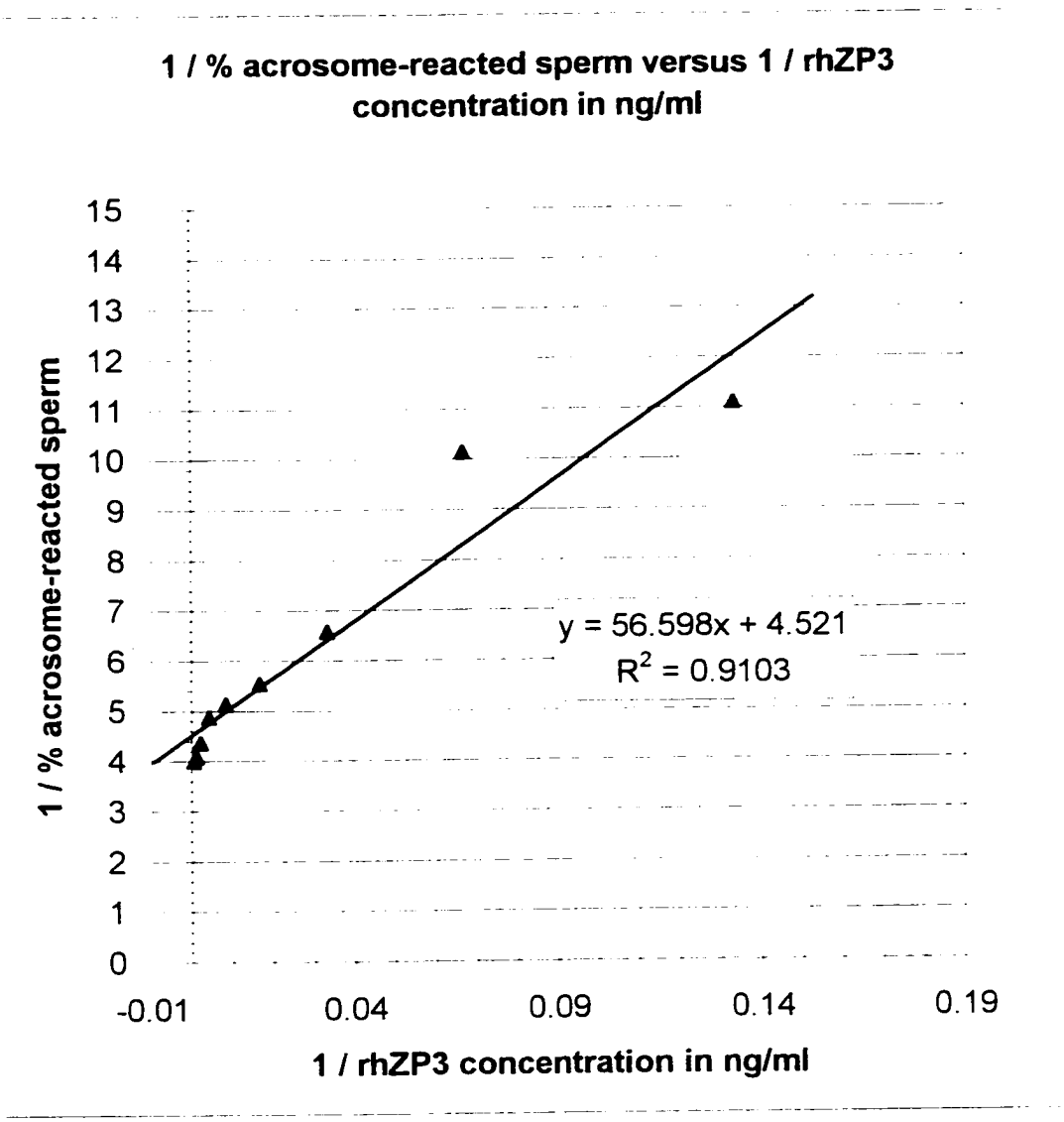

Figure 3. Determination of Maximum Percentage of Acrosome-Reacted Sperm of rhZP3. A direct plot of 1 / % acrosome-reacted sperm versus 1 / rhZP3 concentration in ng/mL to find the maximum % of acrosome-reacted sperm in the assay. The y-interception of the straight line which reads 4.52 which is 1 / % acrosome-reacted sperm and hence give 24.9% of maximum % acrosome-reacted sperm. The method is adopted from Lineweaver-Burk plot.

used in the present study criteria. Pair *t*-test analysis in Table 5 indicates that there was a statistical significance on the calcium ionophore- and rhZP3-treated sperm samples as compared with the non-treated sperm of the same donor. ($p<0.01$). It is therefore interpreted that the percentage of acrosome-reacted sperm when treated with calcium ionophore A23187 and rhZP3 achieved a statistical significance as compared with that of the spontaneous acrosome-reacted sperm in the media with no treatment.

Bonferroni (Versus Control) Multiple-Comparison Test was performed on the percentage of acrosome-reacted sperm treated in the condition of Ham's F-10 (negative), non-transfected PA-1, calcium ionophore A23187, and rhZP3 with the alpha level of 0.05. The statistical analysis showed that the calcium ionophore A23187 and rhZP3 were significantly different from the non-treated Ham's F-10 and non-transfected PA-1. The data also reveals that the non-transfected PA-1 is not different from the negative control.

As depicted in Table 5A, the percentage ratio of Calcium ionophore A23187-treated sperm / Ham's F-10 (negative) is 226.9% ± 116.9% ($p<0.01$): the percentage ratio of rhZP3 / Ham's F-10 (negative) is 198.6% ± 77.2% ($p<0.01$): and, finally the percentage ratio of non-transfected PA-1 (NT) / Ham's F-10 (negative) is 125.5% ± 25.9% ($p<0.01$). In other words, the calcium ionophore-treated sperm increases 226.9% ± 116.9% from its baseline and the rhZp3-treated sperm increases 198.6% ± 77.2% from its baseline. Both increases are statistically significant at a *p*-value of 0.01. In addition, as shown in the data, there is also a default secretion in non-transfected PA-1 that may

Table 5. (A) Percentage acrosome-reacted sperm for Ham's F-10 (negative), non-transfected PA-1, calcium ionophore A23187, and rhZP3 detected by Immunofluorescence techniques of acrosome reaction. The ratio of calcium/negative, ratio of rhZP3/negative, and non-transfected PA-1 (NT)/negative are also included in the table. Student *t*-tests were performed.

n=29, Age 28.3 ± 5.3
3 lots of rhZP3 and 11 donors

	% Negative	% Non-Transfected PA-1	% Calcium Ionophore	% rhZP3
Mean (±SEM)	9.2% (3.8%)	10.2% (2.7%)	19.4% (8.4%)*	18.9% (15.4%)*

	% Ca^{2+}/Neg	rhZP3/Neg	% NT/Neg
Mean (±SEM)	226.9% (116.9%)	198.6% (77.2%)	125.5% (25.9%)

*$p<0.01$

(B) Bonferroni Multiple-Comparison Test (Alpha=0.05) were performed for the Ham's F-10 (negative), Non-transfected PA-1, calcium ionophore A23187, and rhZP3 respectively.

Bonferroni (Versus Control) Multiple-Comparison Test
Responses:Calcium ionophore %, Ham's F-10 (negative) %, Non-Transfected PA-1 %,rhZP3 %
n=29 Alpha=0.050 DF=109 MSE=8.421429E-03 Critical Value=2.431489

Group	Different from groups
Ham's F-10 (negative)	rhZP3, Ca
Non-Transfected PA-1 (NT)	rhZP3, Ca
rhZP3	Negative, NT
Calcium Ionophore (Ca)	Negative, NT

be responsible for the acrosome reaction of the sperm because there is a slight increase from the basal condition. Apparently, this increase does not achieve a statistical significance as indicated in the Bonferroni Multiple-Comparison Test in Table 5B.

In order to determine the percentage of viability for the spermatozoa in the immunofluorescence technique of acrosome reaction. Hoechst 33258 stain was administered to co-stain the spermatozoa in the assay (n=10). Overall speaking, all negative (Ham's F-10 and non-transfected PA-1) and positive (calcium ionophore A23187) controls are more than 95% viability in the immunofluoresence staining (Table 6). Hence, the result from the immunofluoresence technique using FITC-PSA is valid.

F. Transmission Electron Microscopy

This experiment compared the induction of acrosome reaction after 4 hours of capacitation followed by 4 different treatments of the sperm samples: Ham's F-10, non-transfected PA-1 (NT), calcium ionophore, and rhZP3 (Figure 4). A parallel experiment staining the same sperm aliquots with the FITC-PSA was conducted to verify the result of TEM. The reading was done by counting the number of acrosome-reacted sperm in each treatment and compared with that of the FITC-PSA staining. In the calcium ionophore- and rhZP3-treated sperm, the percentages of spermatozoa with disrupted acrosomes or almost complete loss of acrosome were almost 2-fold higher than those detected in the Ham's F-10

Table 6. Viability of Acrosome-Reacted Sperm. The spermatozoa from Ham's F-10 (negative), non-transfected PA-1, calcium ionophore A23187, and rhZP3 are co-stained with Hoechst 33258 stain targeted at the sperm nuclear chromatin for the test of viability of spermatozoa in the analysis of immunofluorescence technique of acrosome reaction.

n=10

	Negative	Non-Transfected PA-1	Calcium Ionophore	rhZP3
% Viability	95.1 (2.6)	98.0 (1.0)	96.5 (2.0)	96.7 (3.2)

Mean (±SEM)

Figure 4. Morphology of Transmission electron microscopy of spermatozoa. (A) A representative spermatozoon from Ham's F-10 (negative) showing intact acrosome; (B) A representative spermatozoon from calcium ionophore-treated sperm showing the loss of acrosomal content from calcium-treated sperm; (C) A representative spermatozoon from rhZP3-treated sperm showing the acrosome-reacted status.

and non-transfected PA-1 in the TEM counting. There is no significant difference between the TEM and FITC-PSA staining for acrosome reaction. This indicates that the FITC-PSA staining may be reliable as the indicator for acrosome reaction. Recombinant hZP3, under the observation of TEM, induces true acrosome reaction that is no difference from the spontaneous acrosome reaction and calcium ionophore-treated.

G. Time course study of capacitation

Table 7 illustrated that the relationship between the hours of capacitation and its corresponding percentage of acrosome-reacted spermatozoa at a condition of 2 million per mL for the sperm concentration and 30 ng/mL for the rhZP3. Figure 5 was a plot of the data in Table 7. It was deduced that at eight-hour period, the percentage of acrosome-reacted spermatozoa reached the highest giving a 22.7%. However, co-staining with the Hoechst 33258 stain showed that there was a drop from 99% live sperm to 86% for 4 hours and 8 hours respectively (data not shown). And another drop to 82% at 12 hours and 66% at the end of 24 hours period (data not shown). This data indicated that the viability of the sperm at 4 hours gave a 99% whose percentage of acrosome reaction was not much lower than that of the 8-hour, 12-hour, and 24 hour incubation periods. However, the viability of 8, 12, and 24-hour incubation batches showed a significant dropped from 99%.

Table 7. Time course study of rhZP3 for Hours of Capacitation after Swim-up. The percentage of acrosome-reacted spermatozoa were recorded as mean ± SEM. The concentration of rhZP3 for this assay was 30 ng/mL with a sperm concentration of 2 million per mL.

n=4

Hours Capacitation	% Acrosome-reacted sperm (Mean)	±SEM
0	8.91%	1.74%
1	9.60%	1.86%
4	16.67%	2.35%
8	22.70%	5.07%
12	21.24%	4.58%
24	19.27%	3.96%

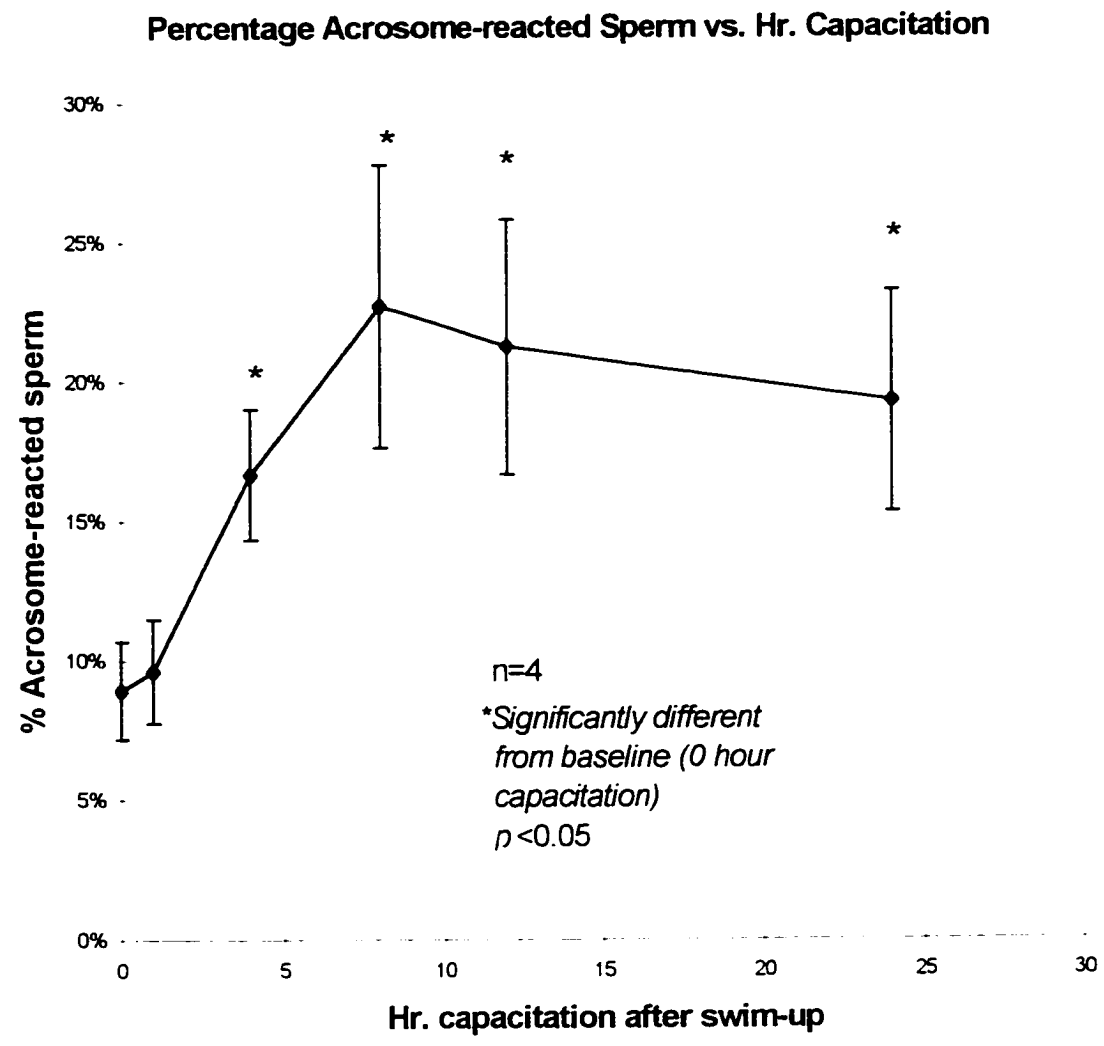

Figure 5. Time-Course Study of Capacitation. A plot of percentage acrosome-reacted sperm versus hours of capacitation after the swim-up. The maximum point is at 8 hours of capacitation after the swim-up which correlates to 22.7% of acrosome-reacted spermatozoa.

H. Varied sperm concentration and rhZP3

Figure 6 indicated spermatozoa were diluted and adjusted through serial dilution. Then each adjusted sperm concentration group was incubated with 500 ng/mL of rhZP3 for 30 minutes. The percentages of acrosome-reacted spermatozoa for each concentration were recorded and a graph of percentage acrosome-reacted sperm in response to 500 Ng/mL rhZP3 against the adjusted spermatozoa concentrations was drawn. The curve showed a hyperbolic relationship between the concentration of spermatozoa reacting to a fixed amount of rhZP3 at 500 ng/mL and its induced acrosome reaction.

I. Motion analysis of rhZP3-treated sperm

In Table 8, the table shows the parameters of the motion analysis of spermatozoa at a concentration of 30 ng/mL rhZP3 for the determination of the status of hyperactivation. The parameters indicated were VSL (Progressive Velocity), VCL (Track Speed), ALH (Lateral Amplitude), LIN (Linearity), Human Hyperactivity. The measured factors were rhZP3-treated, non-transfected PA-1. and Ham's F-10 / 0.5%HSA (no-treatment) media at 0, 1, 2, and 4 hours capacitation after swim-up preparation. Student *t*-tests were performed to compare the rhZP3-treated and non-transfected PA-1 (NT-PA1) treated spermatozoa as well as the rhZP3-treated and Ham's F-10 / 0.5% HSA spermatozoa. There was no difference among the treatment and control groups for each category of parameters. Therefore, rhZP3 does not induce hyperactivation.

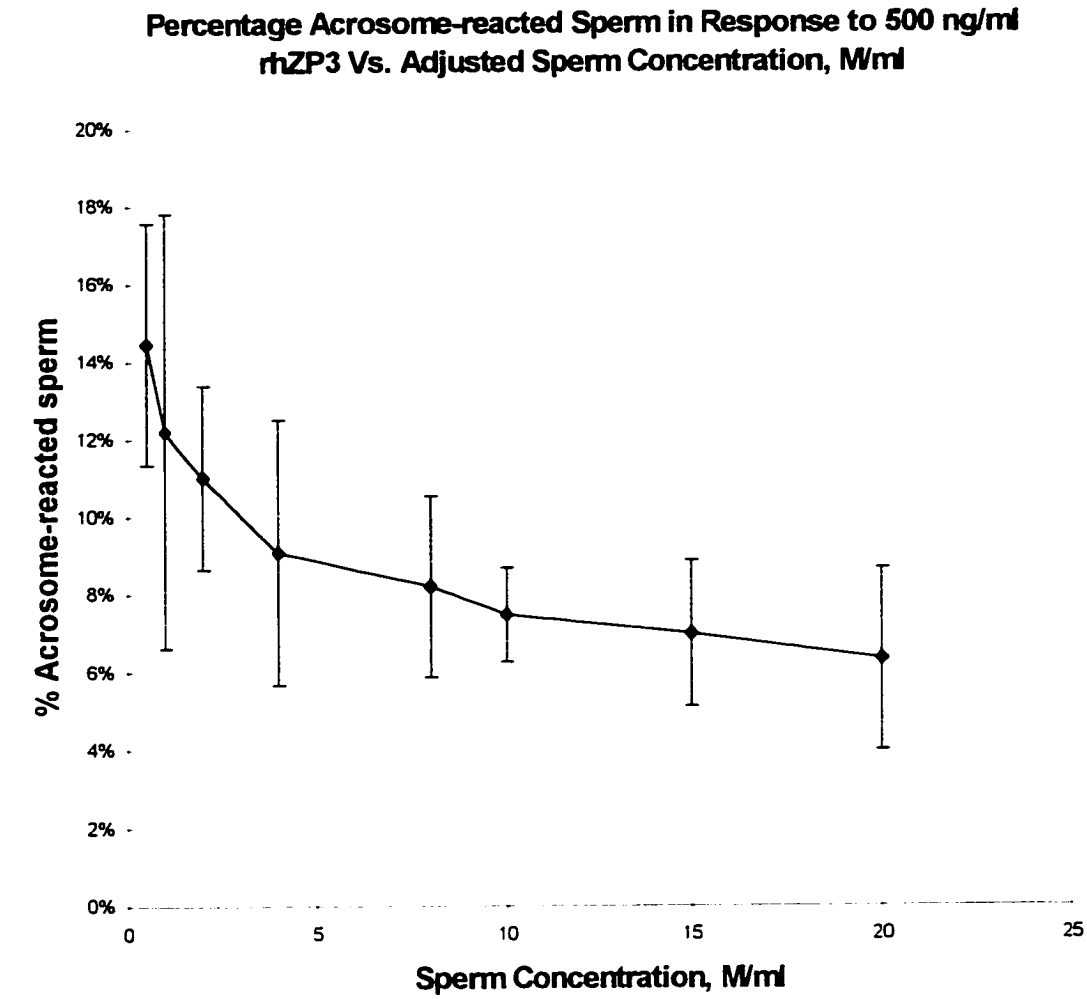

Figure 6. Sperm Concentration Study at 500 ng/mL rhZP3. The concentrations of the spermatozoa were adjusted accordingly before the incubation with 500 ng/mL of rhZP3. The percentages of acrosome-reacted spermatozoa for each concentration were recorded and a graph of percentage acrosome-reacted sperm in response to 500 ng/mL rhZP3 against the adjusted spermatozoa concentrations was drawn.

Table 8. Motion Analysis of rhZP3-Treated Sperm. Motion analysis of spermatozoa in rhZP3-treated, non-transfected PA-1, and Ham's F-10 / 0.5%HSA (no-treatment) media using Hamilton-Thorn Sperm Analyzer for VSL (Progressive Velocity), VCL (Track Speed), ALH (Lateral Amplitude), LIN (Linearity), and Human Hyperactivity at 0, 1, 2, and 4 hours capacitation after swim-up preparation. Student *t*-tests were performed to compare the rhZP3-treated and non-transfected PA-1 (NT-PA1) treated spermatozoa as well as the rhZP3-treated and Ham's F-10 / 0.5% HSA spermatozoa.

n=3

Hours Capaci-tation	VSL	VCL	ALH	LIN	Hyper-activa-tion	
0	62.1 (40.7)	86.8 (36.3)	3.8 (3.3)	64 (22)	4 (2)	rhZP3-treated*
	64.0 (40.3)	87.1 (38.8)	3.9 (3.2)	65 (25)	5 (2)	NT-PA1*
	63.4 (41.3)	87.3 (39.9)	4.1 (2.5)	67 (29)	5 (2)	No-treatment*
1	67.4 (36.6)	81.8 (35.2)	3.3 (1.6)	79 (22)	4 (2)	rhZP3-treated*
	68.1 (35.4)	85.8 (40.2)	4.2 (2.5)	76 (23)	5 (1)	NT-PA1*
	69.5 (39.3)	94.5 (45.0)	4.8 (3.0)	71 (25)	4 (3)	No-treatment*
2	69.2 (36.5)	88.8 (41.4)	4.4 (2.4)	75 (23)	4 (3)	rhZP3-treated*
	67.6 (36.8)	90.1 (39.9)	4.4 (2.7)	72 (26)	5 (2)	NT-PA1*
	74.3 (40.6)	95.9 (49.8)	4.5 (2.5)	75 (24)	5 (2)	No-treatment*
4	54.3 (38.7)	78.7 (45.4)	5.0 (2.3)	64 (26)	4 (1)	rhZP3-treated*
	58.9 (33.7)	75.4 (37.9)	4.2 (2.1)	73 (23)	4 (3)	NT-PA1*
	66.6 (38.6)	86.7 (45.1)	4.6 (2.3)	71 (24)	5 (2)	No-treatment*

*Comparing rhZP3-treated with NT-PA1 and no-treatment (negative) they are not statistically significant at p=0.05.

J. Solubilized zona and rhZP3 in Ham's F-10 and HTF

In comparison of solubilized zona to rhZP3, Table 9 illustrates the percentage of acrosome reaction of rhZP3 is 18.3 ± 1.4 (p<0.05) and solubilized zona is 14.8 ±1.7 (p<0.05) in Ham's F-10 / 0.5% HSA. In contrast, the percentage of acrosome reaction of rhZP3 is 28.2 ± 5.6 (p<0.05) and solubilized zona is 23.3 ± 6.2 (p<0.05) in HTF / 0.5% HSA. It is deduced that the HTF can have more capacitation that leads to more percentage of acrosome reaction. The solubilized zona cannot be compared to rhZP3 in terms of their concentrations because an exact concentration from the solubilized zona is not determined.

K. Oligopeptides of mouse ZP3-1 and human ZP3-6

The present study also evaluates the human sperm with synthetic oligopeptide of mouse and human ZP3. Table 10 illustrates the comparison between ZP3-1, ZP3-6 and rhZP3 with corresponding controls. The mouse ZP3-1 oligopeptide did not induce acrosome reaction significantly above the baseline. However, the human ZP3-6, which is 10 amino acid oligopeptide of hZP3, induces acrosome reaction significantly (p<0.05). The rhZP3 had also induced acrosome reaction significantly from the baseline (p<0.05).

L. G-protein antagonist: Involvement of G-protein in acrosome reaction signaling cascade

Pertussis toxin is the G_i-protein antagonist which is used to coincubte with rhZP3 (Table 11). Percentage of acrosome reaction of pertussis toxin/rhZP3-

Table 9. Solubilized Zona and rhZP3 in Ham's F-10 and HTF. Percentage of acrosome-reacted sperm for the comparison of different exposure to Ham's F-10 / 0.5%Human Serum Albumin (HSA) and Human Tubal Fluid (HTF) /0.5% HSA for the treatments 500 ng/mL rhZP3, solubilized zona (0.5ZP/mL) and controls.

Induced by Ham's F-10 / 0.5%HSA
n=3

	Negative %	Non-Transfect %	Calcium Ionophore %	500 ng/mL rhZP3 %	Solubi-lized zona 0.5ZP/mL %
Mean (SEM)	7.7 (3.2)	9.5 (2.4)	22.5 (4.1)*	18.3 (1.4)*	14.8 (1.7)*

*$p<0.05$

Induced by HTF/0.5%HSA
n=3

	Negative %	Non-Transfect %	Calcium %	500 ng/mL rhZP3 %	Solubi-lized zona 0.5ZP/mL
Mean (SEM)	5.7 (4.8)	8.2 (2.7)	34.7 (5.2)*	28.2 (5.6)*	23.3 (6.2)*

*$p<0.05$

Table 10. Effect of Mouse ZP3-1 and Human ZP3-6 on Acrosome Reaction. Percentage acrosome reacted sperm in the mouse-specific ZP3-1, human-specific ZP3-6 and rhZP3 compared in the level of 30 ng/mL were measured. Appropriate positive control such as calcium ionophore A23187 and negative controls such as the non-transfected PA-1 (NT) and Ham's F-10 media were included.

n=3

	Negative %	NT %	Calcium %
Mean (±SEM)	7.36% (1.56%)	9.69% (2.35%)	17.92% (0.94%)*

	ZP3-1 %	ZP3-6 %	rhZP3 %
Mean (±SEM)	7.30% (2.21%)	14.76% (2.01%)*	14.52% (1.42%)*

$p<0.05$ as compared to negative

treated sperm did not show significantly different from the baseline. The rhZP3-treated sperm, on the other hand, increased significantly from the baseline (p<0.5).

M. Inhibition of G_i-protein by pertussis toxin (PTX) in binding test of Hemizona Assay

Pertussis toxin (PTX), a G_i-protein antagonist, could inhibit the acrosome reaction induced by the rhZP3 induced, G_i-protein mediated signal transduction pathway. Table 12 depicted that there was no significant difference between the HZI of test group (3-hour incubation of 1 μL of 100 ng/mL PTX in the 100 μL sperm droplet followed by 0.5-hr incubation with the 500 ng/mL rhZP3) and control group (3-hour incubation of 1 μL of 100 ng/mL Ham's F-10 / 0.5% HSA in the 100 μL sperm droplet followed by 0.5-hr incubation with the 500 ng/mL rhZP3).

N. Intracellular Ca^{2+} concentration change for rhZP3-treated and potentiation of rhZP3 with physiological and chemical compound

Figure 7 depicts the measurement of intracellular Ca^{2+} concentration change when the sperm samples (n=4) were treated with 5 different lots of rhZP3 that had been previously tested with hemizona assay and immunofluorescence detection of acrosome reaction. There is a change in intracellular Ca^{2+} concentration when 10^{-6} M or 1 nM progesterone was added. However, a high dosage of 4000 ng/mL rhZP3 did not trigger any change in intracellular Ca^{2+} concentration using the FURA-2 Fluorospectroscopy. The potentiation

Table 11. Effect of G-Protein Antagonist on Acrosome Reaction. Sperm samples were co-incubate with 100 ng/mL pertussis toxin (PTX) and 500 ng/mL rhZP3 to block the G-protein dependent signal transduction pathway. Recombinant human ZP3 at 500 ng/mL is included in the assay for comparison. Positive control is calcium ionophore A 23187; negative controls are Ham's F-10 (negative) and non-transfected PA-1 (NT).

n=3

	Negative %	NT %	Calcium %
Mean (±SEM)	8.09 (0.20)	7.95 (0.31)	24.23 (18.12)*

	500 ng/mL rhZP3 %	100 ng/mL PTX followed by 500 ng/mL rhZP3
Mean (±SEM)	19.73 (0.91)*	10.16 (0.42)

*$p<0.01$

Table 12. Effect of Pertussis Toxin on the Sperm-Zona Binding. HZI of test group (3-hour incubation of 1 μL of 100 ng/mL PTX in the 100 μL sperm droplet followed by 0.5-hr incubation with the 500 ng/mL rhZP3) and control group (3-hour incubation of 1 μL of 100 ng/mL Ham's F-10 / 0.5% HSA in the 100 μL sperm droplet followed by 0.5-hr incubation with the 500 ng/mL rhZP3) were shown.

	HZI of 100 ng/mL PTX followed by 500 ng/mL rhZP3	**HZI of 500 ng/mL rhZP3**
Mean (±SEM)	51.4 (3.2)	54.1 (4.2)

n=2 donors x 3 matching pairs of hemizonae

experiment of 4000 ng/mL rhZP3 followed by 10^{-6} M or 1 nM progesterone did not have observable increase from that of 10^{-6} M or 1 nM progesterone alone. Another potentiation experiment was done with the treatment of rhZP3 (15 minutes) followed by 10^{-6} M or 1 nM progesterone (data not shown) did not potentiate observable increase of intracellular calcium different from the 10^{-6} M or 1 nM progesterone alone. Finally, when the sperm medium, Ham's F-10 / 0.5% HSA, was treated with ethyleneglycol-bis (beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), a calcium-chelating agent, followed by the addition of4000 ng/mL rhZP3. There was no observable increase in the intracellular calcium when the sperm populations (n=2) were detected by the FURA-2 Fluorospectroscopy (data not shown).

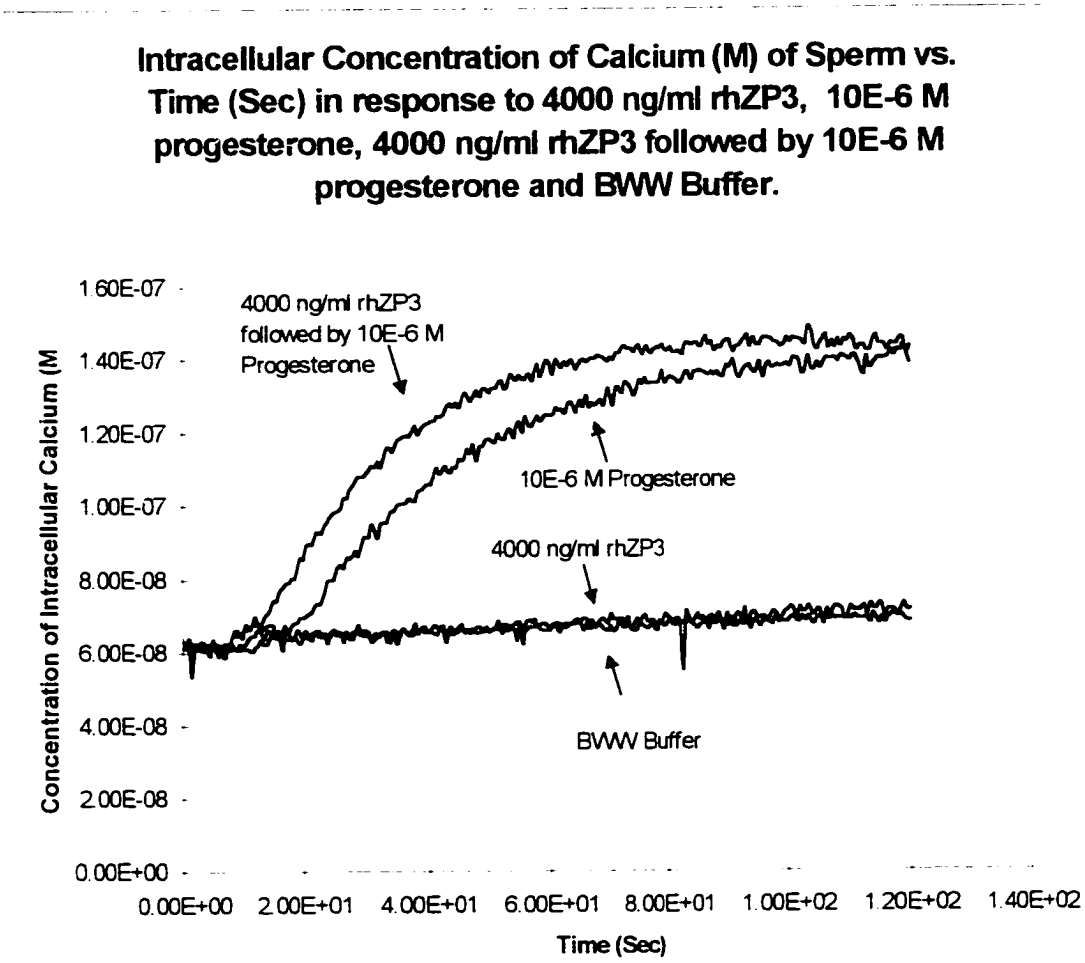

Figure 7. Intracellular Calcium Measurement by Fura-2 Fluorospectroscopy. A measurement of intracellular concentration of calcium in sperm when treated with 5 different lots of purification of rhZP3 that had been previously tested successfully with hemizona assay and immunofluorescence staining for acrosome reaction (n=4). These sperm samples were also exposed to progesterone and BWW buffer.

CHAPTER V

DISCUSSION

In general, this study is to characterize the biological functions of recent expressed and purified rhZP3 in our laboratory (Chi, 1998) which has reported to have a 60% purification of rhZP3 with the MW 62-65 KDa and pI of 3.5-5.3. The evidence is based on the SDS-PAGE and the Western blotting. The data are consistent with the characterization of hZP3 reported by Bercegeay and his colleagues (1995).

Male-factor infertility of unknown etiology has not been fully explored and has remained as one of the most challenging obstacle in the reproductive medicine. Approximately, 30-40% of childless couples results from the male infertility (Wittemer et al., 1996). There is high correlation of infertility in male-factor individuals and the sperm-zona pellucida interaction is frequently observed in these cases (Chan et al., 1991; Honig et al., 1993). With regard to the human system, production of purified glycosylated zona proteins in a biologically active form is difficult to obtain due to technical difficulties (van Duin et al., 1994, Barratt and Hornby, 1995).

The hypothesis of this study is that sperm-oocyte interaction requires zona pellucida protein 3 (ZP3) both as sperm-oocyte binding ligand and acrosome reaction inducer that leads to a signal transduction.

The first specific aim of present study is to characterize the biological activity of the rhZP3 as sperm-binding ligand and acrosome reaction inducer in fertile human males.

The use of microdroplets in the assisted reproductive laboratory has been used for detection for patients with unexplained poor fertilization or even with fertilization failure (Barrett, et al., 1995; Ozgur et al., 1995). Mammalian gamete. including the human's, involves a specific receptor(s) on the sperm surface and glycoproteins of the zona pellucida. An inhibition of the binding in hemizona assay at the concentration levels of 30 ng/mL and 500 ng/mL of rhZP3 ($p<0.01$) was observed and further dose-dependency study of inhibition of rhZP3 in the hemizona assay as observed in the present study might be caused by masking of zona receptors on sperm. Hence rhZP3 may block the binding to intact zona pellucida during incubation. Inhibition was observed to be in a competitive and dose-dependent in a linear fashion for the first 500 ng/mL of rhZP3. However it has a plateau at the HZI=35.8 at 2000 ng/mL rhZP3. This has been consistent with the observation reported by Franken et al. (1996) who observed a linearity of decrease using a solubilized zona pellucida for the inhibition study. Unfortunately. there is no report on the highest inhibition capability of the solubilized zona pellucida protein on the sperm to date. The plateau may be due to many factors. which can only be postulated but not, concluded until further investigations are done. The first possible explanation is that the glycosylation of the rhZP3, which is essential for the primary binding of sperm to oocyte, is not 100% homologous with the physiological hZP3. However, the glycosylation of rhZP3 was able, though not perfect, to act on the tight primary binding. The second possibility responsible for the inhibition might be the inability of the acrosome-reacted sperm to bind to the zona pellucida. It is known that the sperm must be acrosome reacted to complete penetration of zona (Franken et al., 1991a). The exact site of

the acrosome reaction has not been identified and appears to differ between species. In the murine system, only acrosome-intact sperm are able to bind to the zona pellucida and the fertilizing sperm hence undergo acrosome reaction on the surface of the sperm (Saling and Storey; 1979). It is not conclusive enough whether the acrosomal exocytosis on the zona pellucida represents the fertilizing sperm in human (Kopf and Gerton; 1990) because both acrosome-intact and acrosome-reacted sperm can both involved in the initial stages of zona binding to the human zona pellucida (Morales et al. 1989). The electron microscopic study on acrosome-intact, partially reacted and fully reacted sperm revealed that binding of sperm to oocyte could be acrosome-intact and acrosome-reacted sperm in human. However, only fully acrosome-reacted sperm were reported to have penetrated in the inner zona pellucida (Barrett et al., 1995). In the present study, Table 3 indicates the percentage of acrosome-reacted sperm in the rhZP3-treated group is not significantly different from the baseline (negative control) and Table 12 depictes that the rhZP3 still exhibits a binding ligand in the pertussis toxin-blocked acrosome reaction of sperm populations. It is therefore deduced that the rhZP3 is a binding ligand for the sperm-zona interaction.

Binding of the sperm can be viewed from two stages: (a). Upon contact, immediate initial binding of sperm to zona takes place in less than 1 minute for a temporary attachment to the zona. (b). The tight primary binding gives a irreversible and permanent attachment between sperm and zona in a period of more than 2 hours of coincubation of gametes (Franken, et al., 1991a).

Hence we can postulate that acrosome-reacted sperm can associate loosely with the zona by reversible attachment to the outer zona surface which

may be partly dislodged during the washing procedure in the hemizona assay. For the acrosome-intact sperm, it will achieve an irreversible bond with the zona in the outer acrosomal membrane and then undergo the acrosome reaction on the surface of the zona before the exocytosis of acrosome.

Also, the clinical testing has demonstrated a positive correlation between the number of sperm bound to the hemizona and success during *in vivo* fertilization (Franken et al, 1989a and 1989b; Oehninger et al., 1988). It is further postulated the possibility of rhZP3 developed in our laboratory can be used as the diagnostic tool for male-factor patients.

In the present finding of spontaneous acrosome reaction, our data is similar to the result from Singer et al. (1993) but is not parallel to the findings in Goluld *et al* (1985). This indicates that the immunofluorescence method of acrosome reaction varies from laboratory to laboratory and is depending upon many factors affecting the capacitation such as the incubation time, media used, bicarbonate/CO_2 and temperature (Harrison et al. 1996). Also, it varies from populations of donors selected for the experiments (Morales et al., 1994). When the Ham's F-10 / 0.5%HSA media was replaced by the HTF (Human Tubal Fluid) / 0.5%HSA, the percentage of acrosome-reacted sperm increase significantly for the replacement. However, the percentile ratio of increase from the baseline or spontaneous acrosome reaction remains similar. The ratio will eliminate any internal error resulting from the variance in samples. Therefore, the present study will report the acrosome-reacted sperm in rhZP3-, calcium ionophore- and pertussis toxin-treated as percentage ratio increase in the later part of the discussion.

Our acrosome reaction of rhZP3-treated sperm is 198.6% significantly increased from the baseline (n=29; $p<0.01$). Bonferroni Test also indicates a significant different for rhZP3-treated sperm to undergo acrosomal exocytosis compare to that of the baseline and the non-transfected PA-1 secretion ($p<0.05$). When solubilized zona was used for comparison to our rhZP3. there is no difference in their ability to induce acrosome reaction.

Since the glycosylation pattern in human determines the biological activities of hZP3 (Barratt et al.. 1994), the use of rhZP3 from human ovarian teratocarcinoma cells (PA-1) provide a better closeness resembling the glycosylation pattern for hZP3 compare to that from other expression systems. Hence, this is the first of all studies. to date. to demonstrate the glycosylated rhZP3 from human ovarian teratocarcinoma cells (PA-1) is the inducer for the acrosome reaction in human sperm that may be close to the physiological hZP to induce the exocytosis of acrosome. This is also significant because no other human recombinant zona pellucida expressed in other system has shown the inhibition of sperm binding to the zona.

Part of the first specific aim of this study also includes the characterization of the biological activity such as the hyperactivation. dose-dependency. capacitation time and effect of sperm concentration. The conditions tested can be used as the experimental condition for immunofluorescence staining of acrosome reaction in the future study.

Motion analysis of the sperm looking at the parameters of progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH), linearity (LIN) and human hyperactivation parameters indicates no significance in any of parameter

measured when comparing rhZP3-treated to non-transfected PA-1 protein-treated, and no-treatment sperm It is deduced that rhZP3 may not involve in the hyperactivation of the sperm. Many reagents are reported in the literature to induce hyperactivation including fetal bovine serum, FBS. (de Lamirande et al., 1997) which is a biological inducer of hyperactivation. It causes an increase in tyrosine phosphorylation of p105 and p81, the two major sperm phosphotyrosine-containing proteins involved in sperm capacitation. Progesterone and 17OH-progesterone are able to induce a hyperactivation with a rapid, long-lasting, and dose-dependency calcium influx in capacitated human sperm and exocytosis of the acrosomal content (Luconi et al., 1996). Therefore rhZP3 may not involved in the calcium-dependent pathway that hyperactivation has utilized for its signal transduction pathway.

The present study also analyzes the rhZP3 for its dose-dependency factor. At a concentration of 30 ng/mL, the percentage of acrosome-reacted sperm significantly different from the baseline (0 ng/mL rhZP3) ($p<0.05$). It is therefore valid to use 30 ng/mL in the present study. However, there is a technical barrier when the investigator handles the 3×10^5 dilution of rhZP3. Insolubility is a common problem associated with the expression of rhZP3 in other host cell lines. Chapman and Barratt (1997) had reported that the rhZP3 expressed in sf9 insect cells tends to aggregate into insoluble fraction and hence complicate the experimental handling. In our laboratory, our rhZP3-expressed in PA-1 cell line experienced the same fate. Some of our experiments used 500 ng/mL rhZP3 for the hemizona assay and immunofluorescence technique for acrosome reaction is to increase the reaction concentration. Another factor that is worth mentioning is

that the rhZP3 protein lost its structural integrity in a rapid rate when the protein was stored at 4°C for a period of one month (Chi, 1998). An observation of the drop of biological activity to almost non-existing was observed after a two-week storage at 4°C. The structural degradation could be the reason for the drop in biological activity. When the protein was stored in -80°C in 50% glycerol, the biological activity was no different from the fresh. However, the insolubility for the frozen rhZP3 samples is even greater than that in the fresh ones. The aggregation may be due to a hydrophobic region located near the C-terminal of the ZP3 core-protein (Dean, 1992).

The maximum percentage of acrosome-reacted sperm that the present study can achieve is 25% in the Ham's F-10 / 0.5% HSA incubation media that is about 272% increase from the baseline (0 ng/mL).

Capacitation time study of rhZP3 indicates that there is trend of increase in the percentage of acrosome-reacted sperm as the capacitation time prolonged. However, the drop of the percentage acrosome reaction at the end of eight hours can be contributed to the increased death of sperm in the capacitation media due to the metabolic toxic waste excreted as incubation proceeded. The present study choose the 4 hour capacitation as hour choice of incubation time because at this time frame the sperm are 99% alive as compare to 86%, 82% and 66% of live sperm for 8, 12, and 24 hours of capacitation time respectively.

There is gradual drop in the percentage of acrosome-reacted sperm as the concentration of sperm increases for a fix amount of rhZP3. The slopes of the curve get flatter once it reached the 8 million sperm per mL. It is therefore determined that the range of high percentage acrosome reaction is in the range of

0.5-4 million sperm per mL. In addition, the curve demonstrates almost a competitive curve with rhZP3 receptors compete for the fixed amount of rhZP3. This indicates that percentage of acrosome reaction *in vitro* is directly related the number of rhZP3 molecules in contact with the sperm.

Characterization of the rhZP3 hence indicates that the optimal condition for the immunofluorescence staining of acrosome reaction are a concentration of rhZP3 protein of at least 30 ng/mL, capacitation time of 4 hours, and 0.5-4 million sperm per mL.

Further investigation of acrosomal status by transmission electron microscopy of rhZP3-treated sperm indicates that a true acrosome reaction induced by the rhZP3. However, the experiment in the present study comes from only one donor, which must be interpreted with reserve opinion. A partly swollen or diffused matrix of the acrosomal cap was interpreted as the first stage of acrosome reaction, followed by fusions of the plasma membrane with the outer acrosomal membrane, membrane vesiculation (acrosome disruption) and exposure of the inner acrosomal membrane. There is no difference in terms of morphology of the acrosome-reacted spermatozoa in the calcium ionophore-induced and rhZP3-induced sperm populations as observed in the transmission electron microscopy.

When the sperm is treated with the mouse ZP3-1 and human ZP3-6 which are the synthetic oligopeptides of mouse ZP3 and human ZP3 respectively. Our rhZP3 achieved the similar level of acrosome reaction as the human ZP3-6. The fact that mouse ZP3-1 does not induce any acrosome reaction indicate that the peptide backbone of the ZP3 may be responsible for the induction of the

acrosome reaction whereas the glycosylation pattern in rhZP3 may contribute to the primary binding in the sperm-zona interaction. The observation has been consistent with the findings that *O*-linked oligosaccharide chains act as the primary sperm receptor to regulate sperm binding (Bleil and Wassarman, 1980; Florman and Wassarman, 1985; Leyton and Saling 1989). The finding by the present study also merits with the hypothesis that sperm binding activities of the zona pellucida can be associated with internal saccharide core structures (Yurewicz et al., 1991) and with terminal residues (Bleil and Wassarman, 1988). The size and structural features of the oligosaccharide epitope of ZP3 responsible for biological activity appears to be different among species (Noguchi et al., 1992). For example, while terminal carbohydrate residues form the sperm terminal carbohydrate residues from the sperm receptor site in the mouse zona (Bleil and Wassarman, 1988), large internal carbohydrate units regulate sperm binding to pig zonae (Yurewicz et al., 1991).

The second specific aim of the present study is to study the role of the rhZP3 in the signal transduction of the acrosome reaction of sperm. Sperm-associated guanine nucleotide-binding regulatory protein (G-protein) has been shown to be involved during induced acrosomal exocytosis of different species including man (Lee et al., 1992).

The present results illustrate the possible regulatory effect of pertussis toxin on the rhZP-induced acrosome reaction and also highlight the importance of intact acrosomes in the tight primary binding of sperm-zona. A preliminary study of whether pertussis toxin will affect the motion characteristics of the sperm was conducted using Hamilton-Thorn Research Sperm Analyzer (HTR-IVOS) to

examine the parameters progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH), linearity (LIN) and human hyperactivation of pertussis toxin-treated sperm (data not shown). It was found that there is no difference between the motion parameters from the pertussis toxin- and PBS-treated sperm population. This result has been consistent with the laboratory in Dr. Franken's laboratory (1996). Although the pertussis toxin has shown no effect on the motion parameter of the sperm, one should not assume that the capacitation will not be affected because the pertussis toxin in the present study was added after 4 hours of capacitation.

The percentage of acrosome reaction of pertussis toxin-treated sperm followed by the rhZP3 is significantly less than that of the rhZP3-treated sperm alone. The findings as demonstrated from our result have been consistent with several published data using the solubilized zona (Lee et al., 1992; Franken et al., 1996). The human sperm acrosome reaction induced by the rhZP3 appears to be regulated by a sperm-associated G protein of the G_i class. It is consistent with the notion that ZP3-mediated signal transduction through sperm-associated G proteins is a common mechanism by which acrosomal exocytosis is regulated in mammals. Function inactivation of G_i by pertussis toxin does not alter the ability of the sperm to bind to the zona pellucida but inhibits downstream events leading to exocytosis (Florman et al., 1989). In the mouse, pertussis toxin blocks early stages of ZP-induced acrosome reaction (Endo et al., 1987), including the loss of a transmembrane pH gradient that represents one of the earliest transitions of this regulated exocytotic event (Kligman et al., 1991). Our result could be interpreted to support the idea that ZP-induced acrosome reaction is the physiologically

relevant exocytotic event since it is the ZP-induced acrosome reaction and not the spontaneous or calcium ionophore A23187-induced acrosome reaction. The rhZP3 appears to mediate the acrosome reaction through G-protein-mediated signal transduction process that may couple with putative sperm plasma membrane-associated ZP receptors to intracellular effectors (Kopf and Gerton. 1990). The specific function of human sperm G_i protein to induce the acrosome reaction is not clear.

G_i, in other cells, has been implicated in receptor-mediated regulation of Ca^{2+} mobilization (Murayama and Ui, 1985). Brewis and his colleagues (1996) observed a recombinant human zona pellucida glycoprotein 3 expressed in CHO cell induces calcium influx and acrosome reaction in human spermatozoa. Other investigator also found the similar findings in mouse spermatozoa induced by the solubilized mouse zona (Bailey and Storey, 1994). However, extensive literature search reveals only a very few articles reported the calcium influx. In fact, Brewis et al. (1996) is the only paper that reported the calcium influx in the recombinant human zona pellucida-treated sperm in human. Therefore it requires further investigation. Our measurement of intracellular calcium of the human sperm does not reveal any increase of intracellular calcium in rhZP3-treated sperm.

The present evidence supports that G_i-protein mediate the acrosome reaction in human sperm. There is no direct evidence that rhZP3 will couple through the putative sperm plasma membrane-associated ZP receptor/intracellular effectors to induce the mobilization of intracellular Ca^{2+}.

CHAPTER VI

CONCLUSION

1. The recombinant human zona pellucida expressed, isolated and purified in our laboratory acts both as the sperm-zona binding ligand and acrosome reaction inducer.
2. Characterization of the rhZP3 hence indicates that the optimal conditions for the immunofluorescence staining of acrosome reaction are a concentration of rhZP3 protein of at least 30 ng/mL, capacitation time of 4 hours, and 0.5-4 million spermatozoa per mL.
3. The rhZP3 appears to mediate the acrosome reaction through G_i-protein-mediated signal transduction process that may couple with putative sperm plasma membrane-associated ZP receptors to intracellular effectors.
4. Changes in $[Ca^{2+}]_i$ were not detected in populations of sperm with use of FURA-2 spectrofluorometry.

CHAPTER VII

FUTURE APPLICATIONS

What is the future development of rhZP3 that we expressed, isolated and purified in the PA-1 cells. The most important advancement with the rhZP3 development is to have a reliable and repeatable system to establish the production of the protein, in large quantities, for the research and clinical medicine. It can be used to determine the active site of ZP3 and Zp2 binding to spermatozoa. In other words, the external and internal binding of sperm to zona will be studied with the help of rhZP3.

Recombinant human ZP3 can also be used for the study of cell signaling mechanisms in the sperm. The pathways that had been proposed by several researchers could be examined and dissected to explore the possible route of signal transduction pathway used in the sperm.

It is also possible to study the relation of glycosylation pattern of the rhZP3 and the biological activity. Enzymatic digestion of the terminal carbohydrate in a system fashion allows us to understand the importance of glycosylation.

With the development of rhZP3, its three-dimensional protein structure can be determined. The native hZP3 can not have done so because the small quantity and the impurity in the zona pellucida. It is possible to deduce an active site(s) for responsible for primary binding and acrosome reaction.

The other goal for the development of rhZP3 is to explore their use for contraceptive research. Recombinant ZP3 have been used as antigens to

stimulate an immune response. however, the initial enthusiasm for this approach has been discouraged because of the subsequent ovarian dysfunction following immunization in several species (Paterson et al., 1996). Production of defined recombinant zona peptides can be used for epitope mapping studies. Gupta et al. (1996) used such an approach to identify amino acid residues 133-144 and 205-216 of porcine ZP3α (homologous to human ZP1) which are important candidate epitopes for contraceptive design. Such an experimental approach is now possible with the development of rhZP3.

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